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TITLE: Role of Ca++ influx via epidermal TRP ion channels

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14. ABSTRACT

To benefit military veterans with amputations who suffer skin problems on their amputation stumps, this proposal describes mechanistic studies to pave the way for novel methods of improving skin barrier function at the residual limb-prosthetic interface.
Signaling systems in skin will be modulated to increase barrier function, attenuate irritant dermatitis, and characterize the underlying signaling mechanisms so that they can become better targets for treatment.
Progress in year 2 of the funding period is described in this Annual Progress Report.
We maintained all the necessary regulatory approvals from the Durham VA, Duke University IRB and the DoD to conduct the human experimentation. We set up experiments in primary skin cells for mechanical stress, which we found disrupts skin barrier function. We also found that activation of ion channel TRPV4 can re-normalize barrier function of the skin that has been disrupted by mechanical stress. We also found this particular pattern for keratinocytes' regulatory volume decrease, as a surrogate of their capability to moisturize.

15. SUBJECT TERMS

TRP ion channel, epidermis, keratinocyte, mechanical stress, epithelial stress response

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Introduction

The objective of our proposal is to benefit military veterans with amputations who suffer skin problems on their amputation stumps. To fulfill the objective, this proposal describes mechanistic studies to pave the way for novel methods of improving skin barrier function at the residual limb-prosthetic interface.

Rather than attempting to enhance the functions of barrier-enhancing proteins, filaggrin (structural protein in upper epidermal layers) and aquaporins (water channel) by individual targeting, we thought it an attractive (and testable) idea, whether targeting of any of the epidermal transient receptor potential ion (TRP) ion channels could lead to **improved** barrier function and thus better moisturization, thus less irritation, injury and pain. This would be accomplished by affecting both, filaggrin and aquaporin. In keeping with our reasoning, recent insights on activation of TRPA1 and TRPV4 in the skin are supportive of our concept. However, an obstacle toward improved insight is that mammalian skin was not studied in response to mechanical stress in order to wear down the barrier, so that its more rational repair, by targeting specific signaling mechanisms of the epidermis, can be assessed.

Hypothesis

Therefore, the central hypothesis of our proposal is that Ca^{++} influx into epidermal keratinocytes – mediated via TRP ion channels that we intend to activate specifically - controls moisturization and barrier function of the skin by critically influencing filaggrin- and aquaporin function in a cell-autonomous manner, and that activation of these TRP channels can be helpful for repairing a compromised barrier which is caused by injurious mechanical stress.

Specific Aims

(1) to assess skin barrier function and moisturization parameters in response to modulation of TRPV1, 3 and 4 and TRPA1 in normal human skin as it is subjected to mechanical stress.

(2) to assess skin barrier function and moisturization in epidermal cultures derived from military veterans with amputations and stump skin irritation, and to determine the epidermis' response to activation of TRP channels.

Keywords

Amputation stump, irritant dermatitis, epidermis, skin barrier function, TRP channel, aquaporin (AQP) channel, UVB, UVA

Overall Project Summary

Summary of Current Objective

Specific Aim 1	Timeline	Site - investigators	Performance Site
Major Tasks	Months		
Subtask 1a to assess skin barrier function and moisturization in human organotypic skin, setting up basic mechanical injury parameters for subsequent subtasks 2-3; capacitance metrics	1-6	Drs. Lee, Chen, Jin; Liedtke	Duke
ACCOMPLISHED			
Subtask 1b to assess skin barrier function and moisturization in human organotypic skin, setting up basic mechanical injury parameters for subsequent subtasks 2-3; Lucifer yellow metrics	7-12	Drs. Lee, Chen, Jin; Liedtke	Duke
NOT ACCOMPLISHED for Lucifer yellow metrics due to experimental inconsistency. - Capacitance and RVD metrics appear fully sufficient.			
Subtask 2 to assess skin barrier function and moisturization parameters in response to modulation of TRPV1, 3 and 4 and TRPA1 in normal skin, using chemical activation.	13-18	Drs. Lee, Chen, Jin; Liedtke	Duke
ACCOMPLISHED			
Subtask 3 to assess skin barrier function and moisturization parameters in response to modulation of TRPV4 and TRPA1 in normal skin, using UV-light activation	18-24	Drs. Lee, Chen, Jin; Liedtke	Duke
ACCOMPLISHED. UVA-activation of TRPA1 not pursued further because TRPA1 activation was significantly less effective than TRPV4 activation.			
Subtask 4 to assess Filaggrin metrics by Western blotting, immunolabeling, treat human organotypic skin as in Subtask 1	12-24	Drs. Lee, Chen, Jin; Zhang	Duke
NOT ACCOMPLISHED; methodological challenges/ inconsistencies of results.			

Subtask 5 to conduct ultrastructural analysis on organotypic skin, treated as in subtask 1-3	24-36	Drs. Lee, Chen, Jin; Zhang	Duke
NOT ACCOMPLISHED; methodological and organizational challenges			
Subtask 6 to dissociate keratinocytes from human organotypic skin, subject dissociated cells to hypotonic cell swelling; measure regulatory volume decrease and Ca^{++} influx.	24-30	Drs. Lee, Chen, Jin; Liedtke	Duke
ACCOMPLISHED			
Subtask 7 To dissociate keratinocytes from human organotypic skin, subject dissociated cells to hypotonic cell swelling; determine Ca^{++} dependence of regulatory volume decrease by application of specific TRP channel inhibitors for channels TRPV1, TRPV3, TRPV4 and TRPA1.	30-36	Drs. Lee, Chen, Jin; Liedtke	Duke
ACCOMPLISHED			
Milestone Achieved:	12	Define mechanical injury parameters	**DONE**
Milestone Achieved:	18	Define TRP channel contribution to skin moisturization and barrier function	**DONE**
Milestone Achieved:	24	Define contribution of TRPV4 and TRPA1	**DONE**
Milestone Achieved:	24	Assessment of epidermal Filaggrin response	**DONE**
Milestone Achieved:	30	Regulatory volume decrease in response to hypotonicity	**DONE**

Milestone Achieved:	36	Dependence of RVD decrease on skin TRP	**DONE**
Milestone Achieved:	36	Ultrastructural pathology	**NOT DONE**

Specific Aim 2	**Timeline**	**Duke University**	**Durham VA**
Major Tasks	Months		
Subtask 1 IRB protocol review and approval by local IRBs and subsequent review by the ORP	1-4	Hall, Liedtke	Hall, Liedtke
ACCOMPLISHED			
Subtask 2 Enroll 15 patients and 15 healthy controls in the study	5-24	Hall, Liedtke (15)	Hall (15)
NOT ACCOMPLISHED			
Subtask 3 Acquire biopsy specimens (2 from veterans (1 from irritated stump, 1 from unaffected skin), 1 from healthy control) (months 4-24)	5-24	Hall, Liedtke	Hall
Subtask 4 From biopsy samples, prepare specimens for EM	5-24	Chen; Hall, Liedtke	Chen; Hall
Subtask 5 From biopsy samples, derive dissociated keratinocyte cell culture for measurement of regulatory volume decrease as in SA1, Subtask 6/7	5-24	Chen, Lee; Hall, Liedtke, Zhang	Chen, Lee; Hall, Liedtke, Zhang
Subtask 6 From biopsy samples, derive frozen blocks.	5-24	Chen, Lee; Hall, Liedtke, Zhang	Chen, Lee; Hall, Liedtke, Zhang
Subtask 7 From biopsy samples, derive dermal fibroblasts, generate genomic DNA and sequence the *FILAGGRIN* gene.	18-36	Chen, Lee, Jin; Hall, Liedtke, Zhang	Chen, Lee, Jin; Hall, Liedtke, Zhang
Subtask 8 Conduct EM on all specimens (subtask 2)	18-36	Liedtke, Zhang	

Subtask 9 Conduct immunolabeling for TRP channels, aquaporins and FILAGGRIN (samples from subtask 3, 4)	18-36	Chen, Jin; Liedtke, Zhang	
Subtask 10 Conduct qRT-PCR for TRP channels, aquaporins and FILAGGRIN (samples from subtask 3, 4)	18-36	Chen, Lee; Liedtke	
SUBTASKS 3-10: NOT ACCOMPLISHED			
Milestones:	24	Patient-control enrollment	Patient enrollment
	24	Derive their biopsy samples for EM	Derive their biopsy samples for EM
	24	Derive dissociated keratinocytes, RVD testing	Derive dissociated keratinocytes, RVD testing
	36	EM analysis	EM analysis
	36	Immuno-histological analysis	Immuno-histological analysis
	36	mRNA abundance by qRT-PCR	mRNA abundance by qRT-PCR
NONE OF THESE MILESTONES ACHIEVED			

Difficulties in realizing Specific Aim 2 (human subject work):

Challenges in recruiting human subjects have remained throughout the entire period. Our clinical dermatologist-Co-PI Dr Russell Hall believes that the apparent lower prevalence of amputation stump irritant dermatitis in military personnel is possibly due to the improved quality of artificial limbs and re-invigorated stump skin maintenance regimens that are being practiced, based on previous experience. But he also realizes that amputees with stump irritant dermatitis might have felt some frustration with the general level of health care quality in the VA Health System, e.g. wait times and "red tape" obstacles for veterans to receive the high quality health care that they deserve. Therefore, in case amputees had been caught in non-helpful and frustrating circumstances like these, they might have gathered less enthusiasm to participate in research studies that rely on removal of skin biopsies such as the current one. In keeping with this reasoning, PI Dr Liedtke, with his two Duke clinical practices where veterans are being referred to him has not seen a single case of amputee stump irritant dermatitis with predominating pain during the funding period, against a background of an appreciable number of veteran referrals.

Summary of Results

Stress-testing of human artificial skin – accomplished consistent strain >0.54 pertains to the three following subtasks

Specific Aim 1, Subtask 1: *to assess skin barrier function and moisturization in human organotypic skin, setting up basic mechanical injury parameters for subsequent subtasks 2-3; capacitance metrics*

Specific Aim 1, Subtask 3: *to assess skin barrier function and moisturization parameters in response to modulation of TRPV4 and TRPA1 in normal skin, using UV-light activation*

Specific Aim 1, Subtask 4: *to assess Filaggrin metrics by Western blotting, immunolabeling, treat human organotypic skin as in Subtask 1*

In an attempt to optimize application of increased strain, we repeated strain testing, and were able to obtain strains clearly and reliably above 0.5, reaching up to almost 0.55 (Figure 1).

We used human artificial skin as a organotypic human skin substrate, and exerted compression with our bioreactor, evoking the depicted strain.

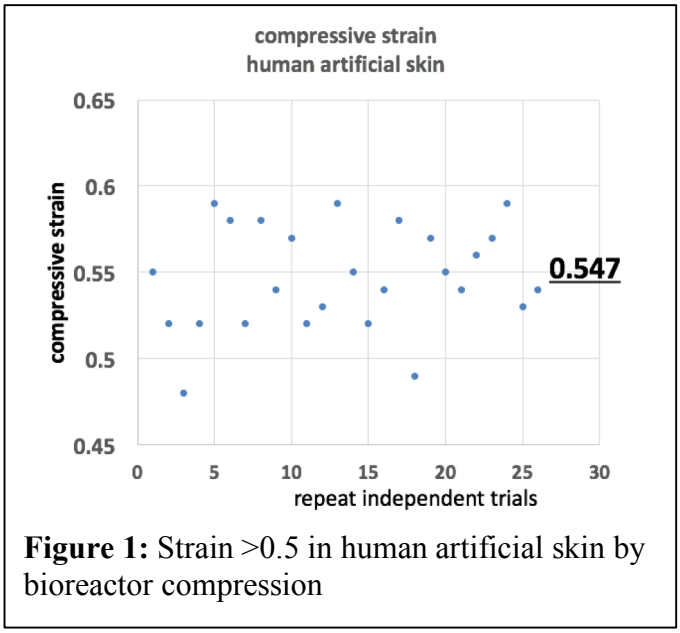


Figure 1: Strain >0.5 in human artificial skin by bioreactor compression

Stress-testing of human artificial skin – barrier improvement by exposure to UVB
pertains to
Specific Aim 1, Subtask 3: *to assess skin barrier function and moisturization parameters in response to modulation of TRPV4 and TRPA1 in normal skin, using UV-light activation*

EpiDermF human artificial skin was grown in culture. We applied mechanical stress at a strain of 0.52 and 0.54, using our modified Flexcell Bioreactor Compression system. We operated it at 1/sec sinusoidal strain for 1h, followed by 1h rest, for a total of 6h, followed by 18h rest, daily repeats, for 5 days.
We applied long-term mechanical stress testing, over 5d, to assess the effect of TRPV4 activation using UVB with the increased exposure protocol (200kJ/cm^2 for 5 min, 10 min preceding mechanical stress). By doing so, we were able to partially rescue the diminished and diminishing capacitance, again encountering some increased variation for the UVB response (Figure 2). In a separate repeat experiment for 48h, at 0.52 strain, we applied TRPV4 blocker GSK205 to culture before the second application, at the 48h time-point, eliminating the improvement caused by UVB. **These data suggest that Ca++ influx via TRPV4 is the relevant signaling mechanism**

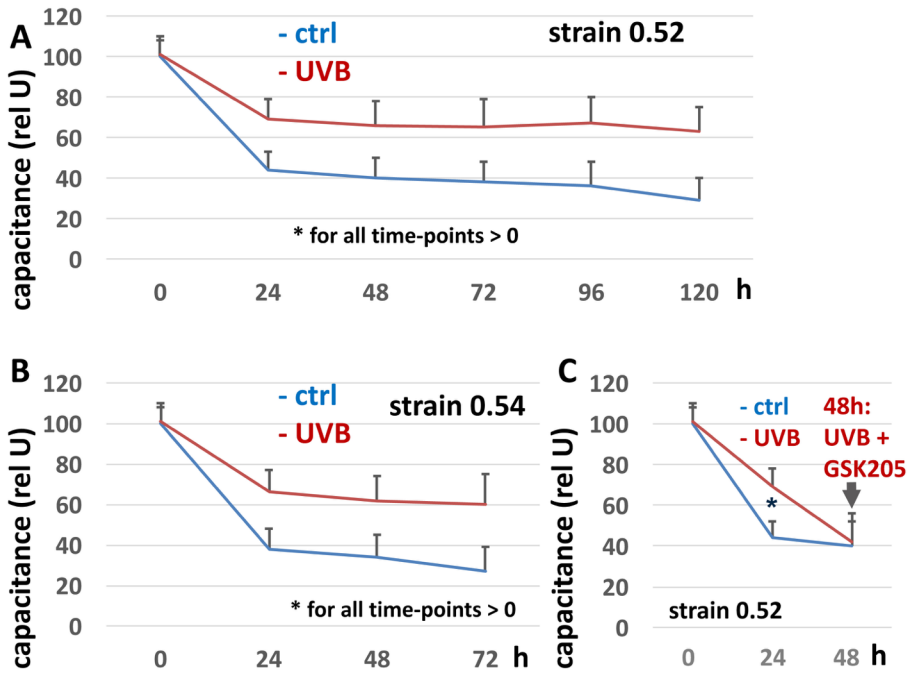


Figure 2: Capacitance decrease of human artificial skin at injurious mechanical strain of 0.52 (A), 0.54 (B), its rescue by application of UVB (A-B), and its dependence on TRPV4 (C). Long-term mechanical compression-stress, to human artificial skin, attenuates capacitance, as a surrogate of barrier function, rescue by application of UVB, shown previously to activate epidermis TRPV4 and increase capacitance. Note mechanical stress here at 0.52 and 0.54 strain level for each application (daily). Experimental n per group n=6; student's t-test for all p-values, which were $p<0.05$ for all time-points starting at 24h.

UVB exposure increases skin capacitance in culture – dependence on TRPV4, not TRPA1

pertains to

Specific Aim 1, Subtask 2: *to assess skin barrier function and moisturization parameters in response to modulation of TRPV1, 3 and 4 and TRPA1 in normal skin, using chemical activation.*

Specific Aim 1, Subtask 3: *to assess skin barrier function and moisturization parameters in response to modulation of TRPV4 and TRPA1 in normal skin, using UV-light activation*

We were able to increase skin capacitance using UVB, as before, with diminished variation. The increase was significantly attenuated by using a TRPV4 inhibitory molecule, not when using a TRPA1 inhibitor (Figure 3).

We used human artificial skin as human skin organotypic substrate. UVB activation of TRPV4 was implemented at energy levels of 200kJ/cm^2 for 5 min, 10 min later followed by measurement of capacitance. Capacitance is a valid surrogate for barrier function. We then used selective TRPV4 inhibitor, GSK205, and TRPA1 inhibitor, A-967079, as indicated in Figure 3. Our results indicate that UVB-mediated capacitance increase depends on TRPV4, not on TRPA1.

Chemical stimulation of TRPV1 (capsaicin and olvanil) and TRPV3 (camphor) did not increase capacitance at all, chemical stimulation of TRPA1 (mustard oil) increased capacitance to non-significant degree.

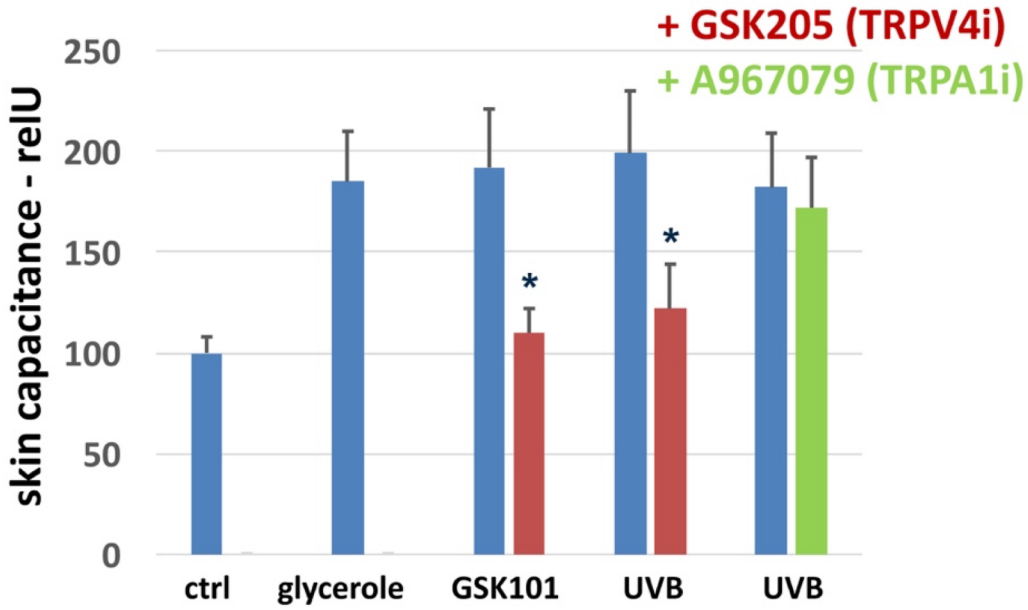


Figure 3: Capacitance increase was accomplished by activation of TRPV4, both chemical and by UVB radiation. Both modes of activation were attenuated, almost completely, by TRPV4-selective inhibitor, GSK205 (10µM). Capacitance increase by UVB was not attenuated by the TRPA1-selective inhibitor, A-967079 (10µM). Thus, capacitance increase via UVB stimulation depends on TRPV4. It does not depend on TRPA1.

Regulatory volume decrease (RVD) of epidermal keratinocytes depends on TRPV4, not TRPV1, TRPV3 or TRPA1
pertains to
Specific Aim 1, Subtask 6: *to dissociate keratinocytes from human organotypic skin, subject dissociated cells to hypotonic cell swelling; measure regulatory volume decrease and Ca^{++} influx.*
Specific Aim 1, Subtask 7: *To dissociate keratinocytes from human organotypic skin, subject dissociated cells to hypotonic cell swelling; determine Ca^{++} dependence of regulatory volume decrease by application of specific TRP channel inhibitors for channels TRPV1, TRPV3, TRPV4 and TRPA1.*

In 2D-tissue culture of keratinocytes, derived from EpidermF, we were able to partially rescue attenuation of RVD after mechanical stress (0.54 strain) by using UVB to activate TRPV4. This partial rescue could be inhibited significantly by TRPV4-selective inhibitory compound GSK205, not by inhibitory compounds selective for TRPV1, SB366791, TRPV3, IPP, and TRPA1, A-967079; all inhibitors used at 10µM (Figure 4).

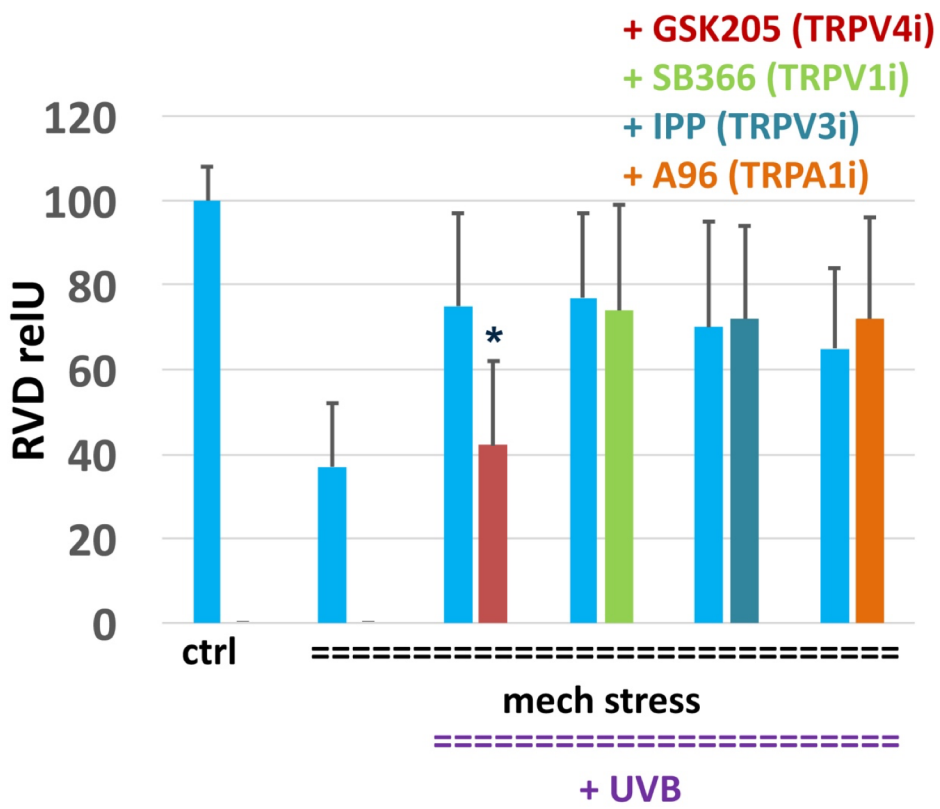


Figure 4: UVB-mediated rescue of impaired RVD, caused by application of mechanical stress (strain 0.54) to human epidermal keratinocytes, depends on TRPV4 ion channels, not on channels TRPV1, TRPV3 or TRPA1.

In addition, we also recorded lack of effect when selectively stimulating (not inhibiting) TRPV1, TRPV3 and TRPA1, yet we recorded consistent rescue of RVD when activating TRPV4.
In 2D primary dissociated human keratinocyte culture, derived from EpidermF, we observed that regulatory volume decrease, in response to 240mosmol/L, was impaired after cells were cultured after mechanically stressing the artificial skin (strain 0.5, sinusoidal stress 0.2Hz, 1h stim/1h rest, 12h stimulation, 12h rest) for 2 days. Activation of TRPV4 with GSK101 could re-normalize the compromised RVD. Activation of TRPV3, TRPV1 and TRPA1 did not have an effect (Figure 5).
Measurement of Ca++ dynamics when subjecting primary dissociated human keratinocytes to hypotonic cell swelling **and** simultaneously to mechanical stress were not informative, showing large variation in signal amplitude, duration and frequency of recurrent Ca++ peaks, very likely because two complex mechano-osmotic stimuli effected the cells at the same time. – In contrast, RVD was consistent as shown in Figures 4 and 5.

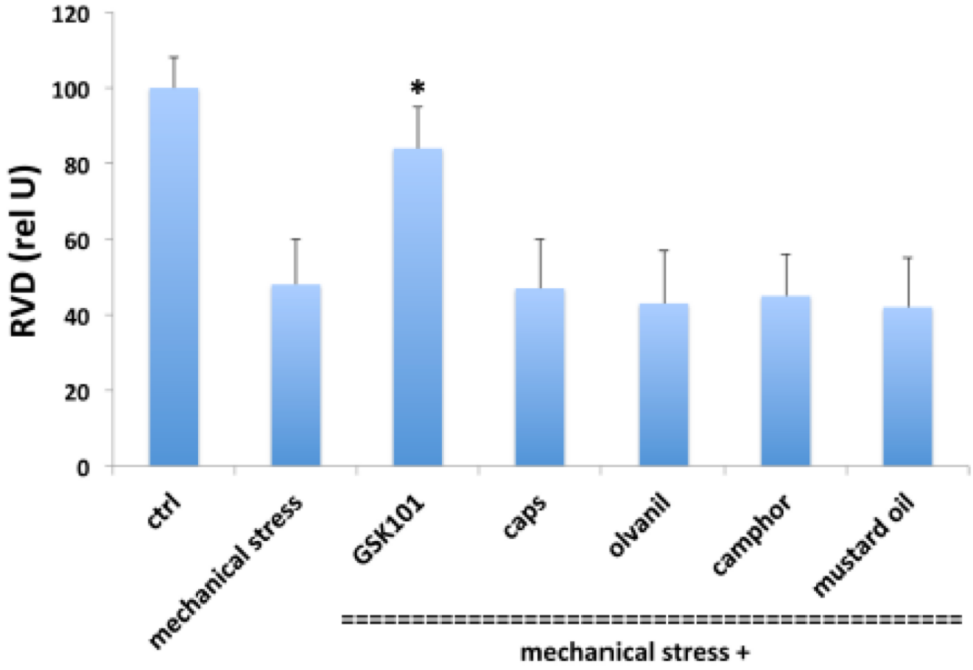


Figure 5: Human keratinocyte regulatory volume decrease is impaired by chronic mechanical stress, can be restored in response to selective stimulation of TRPV4 (GSK101 50nM; * $p<0.05$), not TRPV1, TRPV3 and TRPA1. n=4 experimental replicates were conducted with ≥50 keratinocytes per replicate.

Key Research Accomplishments

- implemented mechanical stress on cultured human artificial skin with a consistent strain >0.5, maximally at >0.54
- stress-testing of human artificial skin could be implemented for up to 240h,
- subjected to chronic mechanical stress, decreased barrier function of human artificial skin could be improved by exposure to UVB, relying on a TRPV4-dependent signaling mechanism
- barrier function of cultured human artificial skin could be improved by UVB exposure, a process dependent on TRPV4, not TRPA1
- osmotic resilience of epidermal keratinocytes depended on TRPV4, not TRPV1, TRPV3 or TRPA1

Conclusion

The stress response of mechanically-stressed human artificial skin can be modulated toward increased resilience by activation of TRPV4 ion channels which are abundantly expressed in epidermal keratinocytes, the main epithelial cell of the mammalian integument. This activation can be accomplished by exposure to TRPV4-activating chemical compounds or to ultraviolet B irradiation.
This conclusion, based on the experimental yield of our proposal, now awaits translation into military-medical practice in order to improve management of amputation-stump irritant dermatitis and other skin health issues of military personnel related to mechanical overload of skin. However, UVB activation of TRPV4 appears to work reliably rather at more robust exposures so that a lower-exposure regime will have to be identified so that the risk of UVB-mediated short- and long-term damage to the integument can be contained, when considering the application of UVB to amputees' stump skin.
During the course of experimentation, we did encounter significant organizational challenges with subject recruitment of military personnel/ veterans. Conducting studies with skin biopsies from these subjects and also phase Ia/b clinical studies in healthy volunteers will be an important next step in order to implement translation. However, the question of dosing UVB at non-damaging levels needs to be addressed. Perhaps fractionated dosing or combination with natural compounds that activate TRPV4 or facilitate UVB-activation will be a constructive approach.

Publications, Presentations

The following **papers** were published / accepted for publication

Chen Y, Fang Q, Wang Z, Zhang JY, MacLeod AS, Hall RP, **Liedtke WB**. TRPV4 ion channel functions as a pruriceptor in epidermal keratinocytes to evoke histaminergic itch. J Biol Chem 2016, 291:10252-62

Kanju P, Chen Y, Lee W, Yeo M, Lee SH, Romac J, Shahid R, Fan P, Gooden DM, Simon SA, Spasojevic I, Mook RA, Liddle RA, Guilak F, **Liedtke WB.** Small molecule dual-inhibitors of TRPV4 and TRPA1 for attenuation of inflammation and pain. Sci Rep 2016, June 1;6:26894. doi: 10.1038/srep26894

Kanju P, **Liedtke W**. Pleiotropic function of TRPV4 ion channels in the central nervous system. Exp Physiol. 2016 Oct 4. doi: 10.1113/EP085790

Moore C, Gupta R, Jordt SE, Chen Y, **Liedtke WB**. Regulation of pain and itch by TRP channels. Neurosci Bulletin 2017, *in press*

Moore C, **Liedtke WB**. Osmomechanical-Sensitive TRPV Channels in Mammals; in Neurobiology of TRP ion channels (CRC Press) 2017, *in press*

Re paper Chen et al.
This paper was accompanied by a Duke University Press Release
https://today.duke.edu/2016/03/itchy
which was picked up by the National Institutes of Health
http://www.niams.nih.gov/news_and_events/Spotlight_on_Research/2016/ion_channel_itch.asp
and news-media world-wide, e.g.
http://www.dailymail.co.uk/news/article-3492763/Time-stop-head-scratching-Scientists-step-close-finding-cure-itching-discovering-skin-protein-causes-irritation.html

Re paper Kanju et al in *Scientific Reports*
The paper was accompanied by a Duke University press release.
https://today.duke.edu/2016/06/doublepain
It generated a lot of public attention, amongst others an interview with National Public Radio (NPR), see also
http://www.wfdd.org/story/duke-researchers-discover-new-class-pain-reliever,
and a widely-accessed posting in the "*Daily Beast*",
http://www.thedailybeast.com/articles/2016/06/01/these-new-painkillers-could-replace-opioids.html
There was also dedicated interest from pain advocacy groups and pain practitioners, e.g. a 1p-full-page coverage in the August issue of "*PainMedicineNews*", the most widely read forum on Pain Medicine in the US and in English-speaking countries.
http://www.painmedicinenews.com/Science-Technology/Article/08-16/Study-'Novel-Compounds'-Coinhibit-Two-TRP-Channels-Related-to-Inflammation-and-Pain/37464

Presentations

* Liedtke W. Translating TRP: Leveraging Basic Insights for Targeted Relief of Pain and Targeted Relief of Pruritus. Plenary Lecture, Translational Pain Meeting, Duke Kunshan University, Kunshan, China; June 23, 2017

* Liedtke W. Pleiotropic function of TRPV4 ion channels. Opening Lecture, Physiology 2016, Joint Meeting of the American Physiological Society and The Physiological Society, Dublin, Ireland; July 29, 2016

* Liedtke W. TRP ion channels as regulators of inflammation and pruritus. Invited Lecture in "Mechanisms of Neuroinflammation and Pruritus", 45th Annual European Society for Dermatology Research Meeting, Rotterdam, The Netherlands; September 10, 2015

* *presentation leading to subsequent publication*

Inventions and Patents

Liedtke W. Activation of TRPV4 ion channel by physical stimuli and critical role for TRPV4 in organ-specific inflammation and itch.
US9290489 (priority 7/6/12; filed 7/5/13; granted 3/22/16)
US9701675 (priority 7/6/12; filed 2/8/16; granted 7/11/17)

Liedtke W. TRPV4 and TRPA1 inhibitors and methods of using the same for organ-specific inflammation and itch. US20160199363A1 (priority 1/9/15, filed 1/11/16, publication 7/14/16); application USPTA - pending (constructive reviews)

Reportable Outcomes and Other Accomplishments

• calcium-permeable ion channel TRPV4 in skin a key regulator-enhancer of barrier function and mechanical stress resilience, can be activated by selective chemicals and UVB exposure

• support from this grant generated results and insights that also contributed to formation of a biotechnology start-up company, TRPblue, founded by the PI, and supported by the Duke University-associated Medblue Incubator of Durham NC

• the PI of this grant was tenured and promoted to Full Professor (with tenure; Departments of Neurology, Anesthesiology and Neurobiology) during the funding period, being awarded this grant was cited as one significant accomplishment

Participants
Co-PIs Wolfgang Liedtke and Russell $Hall_{III}$, Co-I Jennifer Zhang;
Research Scientist Patrick Kanju, Michele Yeo, Whasil Lee, and Research Technician Yingai Jin

Role of Ca^{++} influx via epidermal TRP ion channels

W81XWH-13-1-0299 – Peer-reviewed orthopaedic research program: idea development award
OR120114

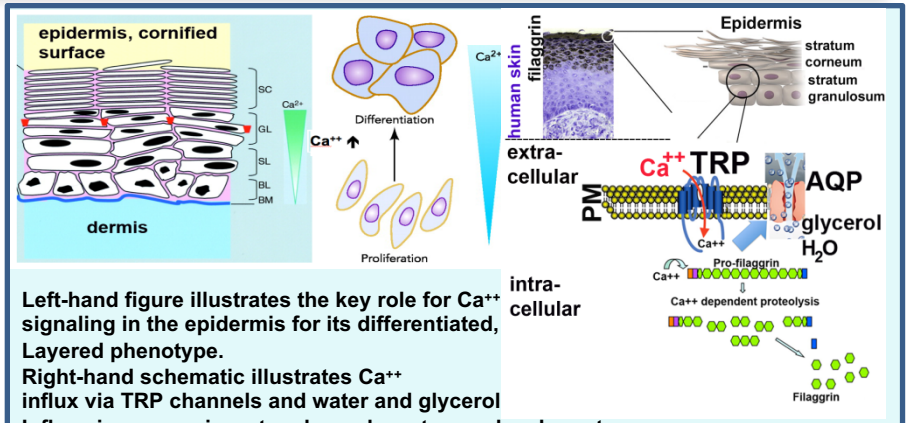
PI: Dr. Wolfgang Liedtke **Org:** Duke University, Durham NC **Award Amount: US$** 775,575.00 (total cost 4y – EWOF granted for y4)

Study Aim(s)

(1) to assess skin barrier function and moisturization parameters in response to modulation of TRPV1, 3 and 4 and TRPA1 in normal human skin as it is subjected to mechanical stress.
(2) to assess skin barrier function and moisturization in epidermal cultures derived from military veterans with amputations and skin irritation of the prosthesis interface of the residual limb, and to determine the epidermis' response to activation of TRP channels.

Approach

We will specifically activate TRP ion channels in human skin keratinocytes and assess whether this has a beneficial effect on skin barrier formation and moisturization state. Human skin organotypic preparations will be used as a generic model (Aim 1),
in addition skin biopsies and cultured keratinocytes from military veterans with skin irritation and dysfunctional barrier and impaired moisturization at the prosthesis interface of the residual limb (Aim 2).



Left-hand figure illustrates the key role for Ca^{++} signaling in the epidermis for its differentiated, Layered phenotype.
Right-hand schematic illustrates Ca^{++} influx via TRP channels and water and glycerol Influx via aquaporin water channels as two molecular gate-ways that will regulate moisturization and barrier function of the epidermis.

Accomplishments: (1) long-term mechanical strain damages skin barrier function which can be repaired by UVB, this mechanism depending on TRPV4; (2) capacitance increase in hu artificial skin in response to UVB, depending on TRPV4, not TRPA1; (3) attenuation of RVD in skin cells in response to mechanical stress repaired by UVB, depends on TRPV4.

Timeline and Cost

Activities CY*	14	15	16	17
Characterize mechanical stress response in cultured human skin				
Modulate mechanical stress response in cultured human skin				
Assess skin barrier function in epi-dermal cultures from mil veterans				
To define the response of cultured skin cells from military veterans to TRP channel activation				
Estimated Budget ($K)	**$166**	**$166**	**$166**	

** showing CY 17 relating to the requested EWOF*

Updated: December 14, 2017

Goals/Milestones

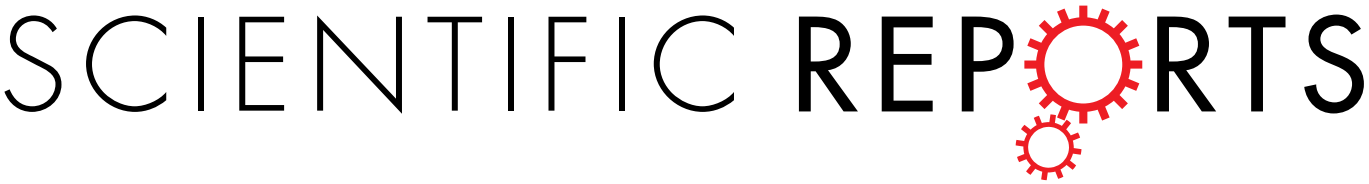
☐ Characterize and modulate mechanical stress response in cultured human artificial skin. **Characterized this response.**
☐ Assess and modulate skin barrier function in epidermal samples and cultures from military veterans with irritant dermatitis of their residual limb and healthy controls. **Maintained regulatory approval, subject recruitment remained challenging throughout.**

Comments/Challenges/Issues/Concerns

- Established role of TRPV4 ion channels as targets. Activation of TRPV4 in skin keratinocytes can attenuate damage to barrier and RVD dysfunction that is caused by injurious mechanical stress. Challenges to enroll military veterans remained.

Budget Expenditure to Date

Projected Expenditure: $775,646
Actual Expenditure: $775,675.92 ($496,237.84 direct cost)



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Small molecule dual-inhibitors of TRPV4 and TRPA1 for attenuation of inflammation and pain

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TRPV4 ion channels represent osmo-mechano-TRP channels with pleiotropic function and widespread expression. One of the critical functions of TRPV4 in this spectrum is its involvement in pain and inflammation. However, few small-molecule inhibitors of TRPV4 are available. Here we developed TRPV4-inhibitory molecules based on modifications of a known TRPV4-selective tool-compound, GSK205. We not only increased TRPV4-inhibitory potency, but surprisingly also generated two compounds that potently co-inhibit TRPA1, known to function as chemical sensor of noxious and irritant signaling. We demonstrate TRPV4 inhibition by these compounds in primary cells with known TRPV4 expression - articular chondrocytes and astrocytes. Importantly, our novel compounds attenuate pain behavior in a trigeminal irritant pain model that is known to rely on TRPV4 and TRPA1. Furthermore, our novel dual-channel blocker inhibited inflammation and pain-associated behavior in a model of acute pancreatitis – known to also rely on TRPV4 and TRPA1. Our results illustrate proof of a novel concept inherent in our prototype compounds of a drug that targets two functionally-related TRP channels, and thus can be used to combat isoforms of pain and inflammation *in-vivo* that involve more than one TRP channel. This approach could provide a novel paradigm for treating other relevant health conditions.

Transient receptor potential Vanilloid 4 (TRPV4) ion channels were initially discovered as osmotically-activated channels[1,2]. Discussing the channel's possible role as mechanosensor, and its expression in sensory neurons in the trigeminal and dorsal root ganglion[1,3,4], led to postulation and eventual experimental validation of a possible function in pain sensing and signaling[1,3–5]. This medically-relevant role was corroborated over time[6–15], as was the mechano-sensory role of TRPV4[11,16–20]. The pro-nociceptive prostanoid PGE2, activation of PAR-2 signaling, inflammation and nerve injury were found to augment TRPV4-mediated pain signaling in various systems[5,6,9,12,21,22], including a novel model of temporo-mandibular joint (TMJ) pain[14]. In a shift of paradigm, TRPV4 was found to function as a relevant sensing molecule in epidermal keratinocytes for UVB overexposure[15]. UVB-exposed keratinocytes, depending on their TRPV4 expression and signaling, were functioning as organismal pain generators, supported by the finding that deletion of *Trpv4* exclusively in these cells sufficed to greatly attenuate the organismal pain response. TRPV4 was also found to play a role in visceral pain, e.g. of the colon and pancreas[7,8,18,23–25], the latter two conditions also co-involving TRPA1[8,24,26–28]. The co-involvement of TRPV4 and TRPA1 was also noted in our TMJ model[14], as well as in formalin-mediated irritant pain of the trigeminal territory, which serves as a generic model of cranio-facial pain[13].

Importantly, blocking TRPV4 with selective inhibitors shows similar results as those obtained with genetic knockouts[13,14,25,29–34], particular in models of TMJ pain or formalin-induced trigeminal formalin pain[13,14]. These findings suggest that TRPV4 could serve as a critical pain target, thus incentivizing the development of more potent and selective small-molecule inhibitors as new clinically-relevant therapeutic drugs. This direction has

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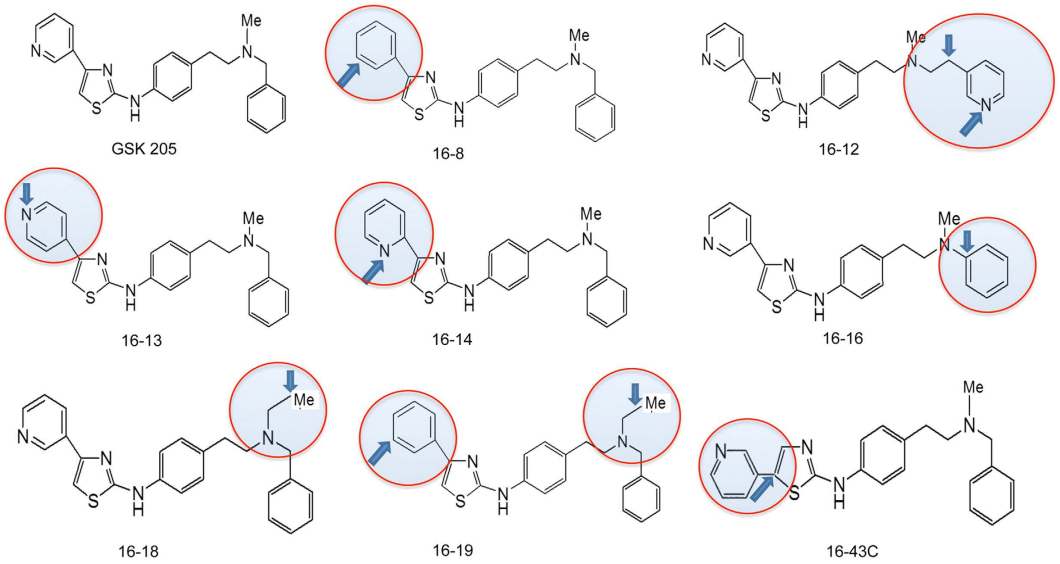

Figure 1. Modifications of tool compound GSK205 for improved targeting of TRPV4. The synthesized compounds differed in the highlighted part of the molecule, changed residue indicated with arrow. Compound 16-19 compound was synthesized to incorporate two modifications from two compounds, 16-8 and 16-18, found most potent in anti-TRPV4 screening assays (see Fig. 2).

advantageous features because genetic approaches are currently limited to experimental conditions and TRPV4 inhibitors are not yet clinically available

The goal of this study was to develop TRPV4 inhibitors with increased potency over a previously used tool compound, GSK205[32–34]. Our results indicate that we have successfully developed compounds with significantly increased TRPV4-inhibitory potency as compared to the tool compound. Interestingly, our approach led to the development of two novel inhibitor molecules that simultaneously target TRPV4 and TRPA1, a potentially advantageous property that we successfully applied in two exemplary *in-vivo* preclinical models of pain, irritation and inflammation.

Results

Chemical synthesis of GSK205 derivatives and assessment of their TRPV4-inhibitory potency in cell-based assays. We modified compound GSK205 by generating 7 primary modifications, as shown in Fig. 1. One additional compound (16-19) that had the combined respective modifications of the two most potent compounds, as defined in primary screens, was also synthesized. We assessed TRPV4-inhibitory potency of these synthetic compounds in a Ca^{++} imaging assay in neuronal 2a (N2a) permanent tissue culture cells with directed expression of mammalian (rat) TRPV4. TRPV4 channels were stimulated with a selective activator compound, GSK1016790A (GSK101), used at 5 nM. For first round assessment, all TRPV4-inhibitory compounds were used at 5 μM (Fig. 2A). Compound 16-43C did not inhibit Ca^{++} influx, and its effect was similar to vehicle control. All other compounds inhibited TRPV4-mediated Ca^{++} influx, with compounds 16-8 and 16-18 emerging as the two most potent. Compound 16-19 which incorporated the modifications of both 16-8 and 16-18, was also effective in inhibiting TRPV4-mediated currents. However, we did not find a significant difference between compound 16-19 and 16-8, both of which virtually eliminated Ca^{++} influx.

We then conducted more detailed dose-response assessments for compounds 16-8, 16-18 and 16-19, which yielded an IC_{50} of 0.45, 0.81 and 0.59 μM, respectively, vs. an IC_{50} of 4.19 μM for parental compound GSK205. These findings represent an increased potency of the GSK205-derivative compounds by approximately 10-fold for 16-8, 8-fold for 16-19 and 5-fold for 16-18. Surprisingly, compound 16-19 was not significantly more potent than 16-8, whereas 16-8 was more potent than 16-18. Based on these results, we tested 16-8 and 16-19 vs GSK205 (as a control) in patch-clamp studies (Fig. 3). Our results indicate significantly increased potency of compounds 16-8 and 16-19 as compared to the parental molecule, GSK205 (all applied at 5 μM) in attenuating TRPV4-mediated currents.

We next decided to assess potency of the most potent compound 16-8 vs. parental compound GKS205 in two types of primary cells that are known to express TRPV4 and in which TRPV4 function has been demonstrated in a relevant biological context. We examined articular chondrocytes, which have prominent TRPV4 expression, where TRPV4 serves as the mechanotransducer of physiologic mechanical loads to regulate the cells' anabolic response, and thus tissue homeostasis, in cartilage[19]. In addition, we studied brain astrocytes, where TRPV4 expression and relevant function has been previously demonstrated in regulating astrocyte cellular edema, in the coupling of neuronal activity to cerebral blood flow, and in mediating CNS traumatic injury[35–37]. Fulfilling our main objective, in both cell types we observed significantly increased potency of compound 16-8 as compared to the parental molecule, GSK205 (Fig. 4). Evaluation of the inhibitory potency of GSK205 derivatives in these primary cells, which express functional and biologically-relevant TRPV4 without

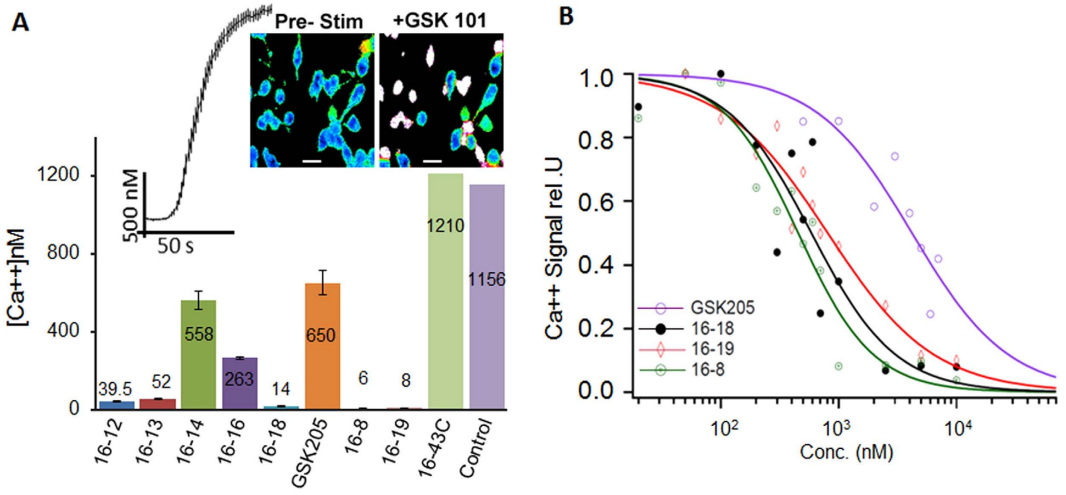

Figure 2. Assessment of 16-… compounds in N2a cells with directed expression of TRPV4. (**A**) Ca^{++} imaging screening of all compounds in N2A cells with directed expression of TRPV4 (rat). The cells were stimulated with TRPV4-selective activator compound, GSK101 (5 nM) in the presence of 5 μM of the respective inhibitor. The number on each bar corresponds to average peak ΔCa^{++} concentrations in ≈100 cells. Inset: micrographs of pseudo-colored cells before and after activation with 5nM GSK101, in addition note the corresponding time course of the averaged Ca^{++} signal (fura-2 Ca^{++} imaging). Except for compound 16-43C, the difference to vehicle control reach the level of statistical significance $p < 0.01$ (one-way ANOVA). **(B)** Dose-response of the most potent, "winner" compounds in TRPV4-expressing N2a cells. The IC_{50} were; 0.45 ± 0.05 μM (16-8), 0.59 ± 0.12 μM (16-18), 0.81 ± 0.1 μM (16-19), 4.19 ± 0.71 μM (GSK205). Plot generated from averaged peak ΔCa^{++} concentration of ≥75 cells per data-point.

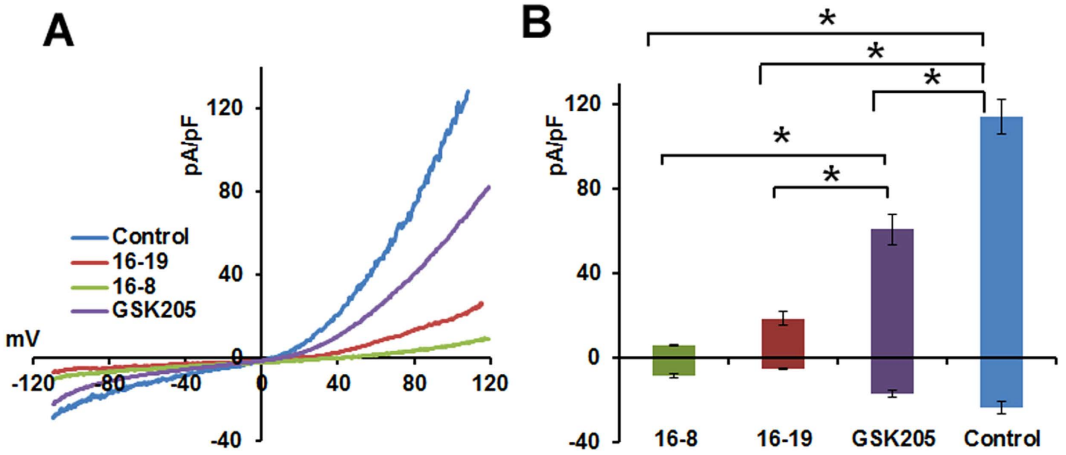

Figure 3. TRPV4 channel inhibition by compounds 16-8 and 16-19 – patch-clamp e-phys. (**A**) Current-voltage relationship of TRPV4-mediated currents after activation with 5 nM GSK101. Recordings were performed in TRPV4-GFP+ N2a cells. The representative traces represent an average of ≈12 sweeps. In all experiments, cells were pre-incubated with the respective compound (5 μM) for 5 minutes. (**B**) Average current densities at −100mV/+100 mV were significantly diminished by inhibitors (*$P < 0.05$; one-way ANOVA; $n \geq 5$ cells/group).

directed over-expression of the channel (Fig. 4), directly corroborate the findings of more basic studies using heterologously TRPV4-overexpressing immortalized cell lines (Fig. 2), strongly supporting our conclusions on the increased potency of the newly derived compounds. Taken together, we identified compound 16-8 as a TRPV4-inhibitory compound with sub-micromolar potency in heterologous systems, with approximately a 10-fold increase in potency as compared to its parental molecule, GSK205. Moreover, 16-8 proved more effective in TRPV4-expressing primary skeletal and CNS-derived cells. However, the rational modification to compound 16-19, intended to further enhance potency, did not yield the intended effect.

Before testing these compounds in relevant *in-vivo* animal models, we next tested their cellular toxicity as well as the specificity of these compounds against other selected TRP ion channels.

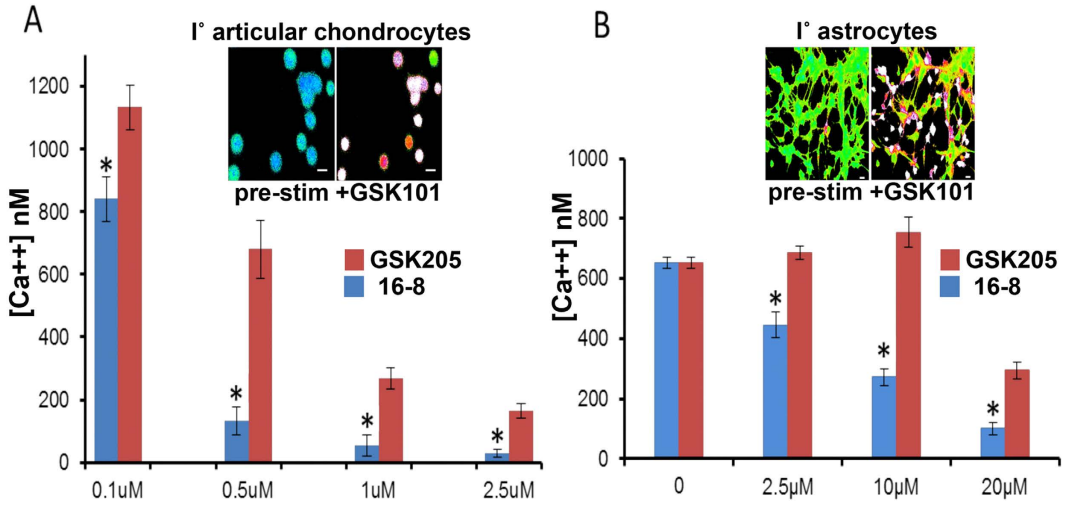

Figure 4. Compound 16-8 inhibits TRPV4 in I° cells more potently than GSK205. (A) I° articular chondrocytes (pig); dose-response comparison between the most potent compound, 16-8, and GSK205 in response to stimulation with 5 nM GSK101. Inset: Chondrocytes responding to activation with GSK101, fura-2 Ca^{++} imaging; right-hand image taken at 5 sec after GSK101 application. 16-8 was significantly more potent than GSK205 (mean ± SEM, n = 6 independent expts, n ≥ 25 cells/expt; *p < 0.05, t-test). Ordinate shows average peak ΔCa^{++} concentrations. **(B)** I° astrocytes (rat); dose-response comparison between 16-8 and GSK205 in response to 5 nM GSK101. Inset: Astrocytes responding to activation with GSK101; right-hand image taken at 5 sec after GSK101 application (mean ± SEM, n = 5 independent expts, n ≥ 200 cells/expt; *p < 0.05, t-test). Ordinate shows average peak ΔCa^{++} concentrations.

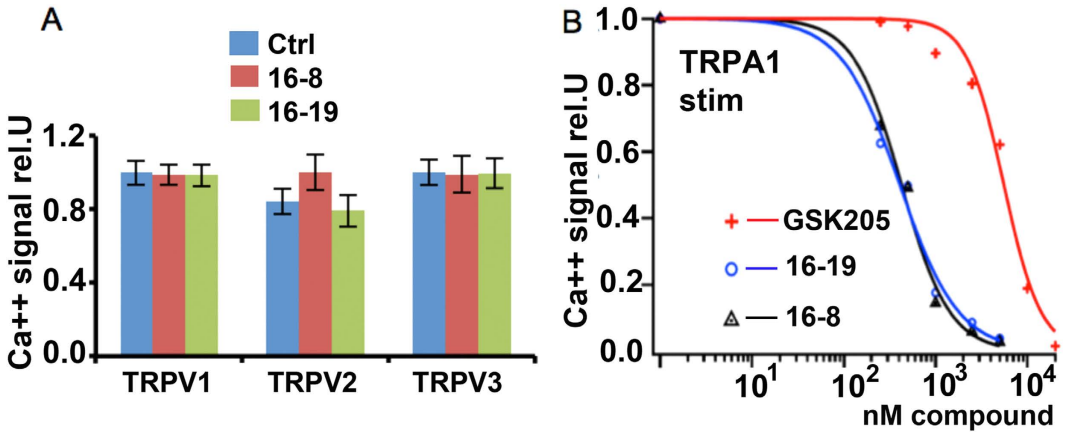

Figure 5. Compounds 16-8 and 16-19 also potently inhibit TRPA1, not TRPV1-3. (A) Specificity vs TRPV1-3. Both 16-8 and 16-19 (5 μM each) compounds did not inhibit TRPV1, −2 or −3 channels (all mouse isoforms), directed over-expression in N2a cells and subsequent Ca^{++} imaging. Mean±SEM is shown, ≥100 cells per condition. **(B)** Dose-dependent inhibition of TRPA1 (mouse, directed expression in N2a cells) by GSK 205, 16-8 and 16-19, activation with 100 μM mustard oil, resulting in IC_{50} of 5.56 ± 0.4 μM (GSK205), 0.41 ± 0.37 μM (16-19), 0.43 ± 0.3 μM (16-8). Plot generated from averaged peak ΔCa^{++} concentration of ≥75 cells per data-point.

Novel TRPV4 inhibitors are selective, with benign toxicity profile, yet display potent inhibition of TRPA1. In heterologously transfected permanent N2a cells, we did not observe inhibitory potency of compounds 16-8 or 16-19 toward TRPV1, TRPV2 and TRPV3 (Fig. 5A). However, we made the unexpected discovery of sub-micromolar inhibitory potency vs TRPA1 for compounds 16-8 and 16-19, micromolar potency for GSK205, and, remarkably, no significant activity for compound 16-18 (Fig. 5B). We recorded IC_{50} of 0.41, 0.43, 5.56 μM and >25 μM for compounds 16-8, 16-19, GSK205 and 16-18, respectively.

In terms of cellular toxicity, we found first evidence of toxicity using a sensitive cell viability assay over a time-course of 2 days, at 20 μM, and more pronounced effects at 40 μM (Fig. 6).

Taken together, our results indicate that compounds 16-8 and 16-19 also inhibit TRPA1 at sub-micromolar potency, and their cellular toxicity vs their inhibitory potency against TRPV4/TRPA1 ranges at a factor of 50–100.

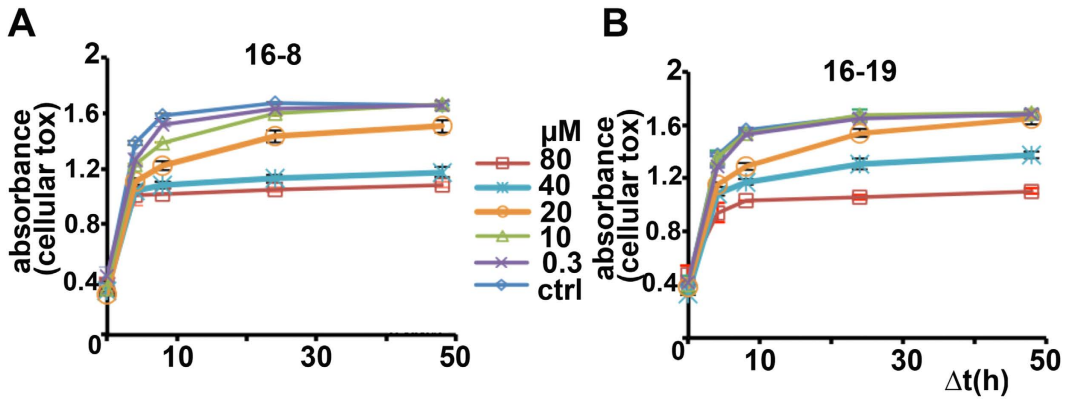

Figure 6. Cellular toxicity studies of compounds 16-8 and 16-19. N2a cells were subjected to increasing concentrations of compounds 16-8 and 16-19, resulting cell viability was analyzed for the next 48 h. **(A)** Time course of cell viability in the presence of various concentrations of 16-8. Note clear reduction at 40 and 80 μM. **(B)** As in (**A**), for compound 16-19, with similar outcome. Representative result of 2 independent experiments.

Assessment of specificity of 16-8, 16-18 and 16-19 against a wider spectrum of receptors and ion channels will be the subject of dedicated future studies directed toward translation of these compounds to the clinic.

We next evaluated these compounds to an *in-vivo* model of irritant pain known to rely on both, TRPV4 and TRPA1.

TRPV4/TRPA1 dual-inhibitors are effective in containing trigeminal irritant pain. We have previously described TRPV4 as a cellular receptor for formalin[13], and demonstrated its involvement in formalin irritant-evoked pain behavior, with a focus on trigeminally-mediated irritant pain behavior. In this recent study, we also demonstrate the co-contribution of TRPV4 together with TRPA1 in trigeminal formalin-evoked pain. We also showed the effective attenuation of trigeminal formalin-evoked pain behavior using GSK205 in a dose-dependent manner. Moreover, we found irritant pain behavior in response to selective activation of TRPV4 in the trigeminal territory, which was blocked by GSK205, and the absence of such an effect in *Trpv4*$^{-/-}$ pan-null mice.

With this pertinent background as a rationale, we applied TRPV4/TRPA1 dual-inhibitory compounds 16-8 and 16-19 systemically, using GSK205 as control, at 10 mg/kg dosage. We prioritized the dual-inhibitors over testing of compound 16-18 (TRPV4-only inhibitor) because (i) the trigeminal formalin pain model relies on both TRPV4 and TRPA1, (ii) we intended to develop TRPV4/TRPA1 dual-inhibitory molecules toward translational use in the first place. None of the compounds were effective at significantly diminishing pain behavior in the early phase after formalin whisker-pad injection, which represents an acute chemical tissue injury pain. In the delayed phase, which is understood as neurally-mediated pain indicative of early maladaptive neural plasticity, there was a significant attenuation of formalin-evoked pain behavior in response to compound 16-8 and 16-19, with compound 16-8 diminishing pain behavior at a remarkable >50%[13], and compound 16-19 also showing a robust effect (Fig. 7A,B). Of note, at 10 mg/kg systemic application, there was no significant effect of GSK205, which was effective previously in a dose-dependent manner when applied by intradermal injection[13]. Thus, compounds 16-8 and 16-19, upon systemic application, effectively attenuate the late, neurally-mediated phase of trigeminal formalin pain, and these compounds are more potent *in-vivo* than their parental compound, GSK205.

In view of these *in-vivo* findings, taken together with the results from heterologous cellular expression systems that indicate an additional TRPA1-inhibitory effect of compounds 16-8 and 16-19, we decided to assess effectiveness of these compounds in a setting of genetically-encoded absence of *Trpv4* (*Trpv4*$^{-/-}$ mouse), in order to better define their TRPA1-inhibitory potency *in-vivo*. We observed significant residual irritant-pain behavior in all phases of the formalin model in *Trpv4*$^{-/-}$ mice, consistent with our previous report[13] (Fig. 7C,D). Immediate-phase pain behavior was virtually eliminated with compounds 16-8 and 16-19, both applied again at 10 mg/kg body-weight. Late-phase pain behavior was strikingly reduced when applying compound 16-8, and still significantly reduced vs vehicle-treated *Trpv4*$^{-/-}$ when applying compound 16-19, although not as potently as 16-8. A reference TRPA1-inhibitory compound, A967079, was used at 25 mg/kg body weight as a positive control to inhibit TRPA1, based on a previous report[38]. Reduction of pain behavior in *Trpv4*$^{-/-}$ mice was striking, more than 50% in the late neural phase. We noted equal potency of compound 16-8 at 10 mg/kg body weight vs. reference TRPA1-inhibitory compound A967079 at 25 mg/kg body weight, both reducing formalin-evoked trigeminal pain behavior to similarly robust degree (Fig. 7C,D). We conclude that compound 16-8 also functions as a potent TRPA1-inhibitor in an *in-vivo* irritant pain model specifically designed to assess the contribution of TRPA1 to trigeminal irritant pain, and at least as potent as an established reference TRPA1-antagonistic compound.

Potent TRPV4/TRPA1 dual-inhibitor, 16-8, is effective at controlling inflammation and pain in acute pancreatitis. These findings define compound 16-8 as a potent TRPV4/TRPA1 dual-inhibitor molecule, based on cell-based and live-animal results. We therefore decided to test it in a more specific preclinical pain model that relies on both, TRPV4 and TRPA1, in order to establish proof-of-principle that a dual-inhibitor can effectively treat pain and inflammation in pancreatitis. We used a pancreatitis model because it provides high

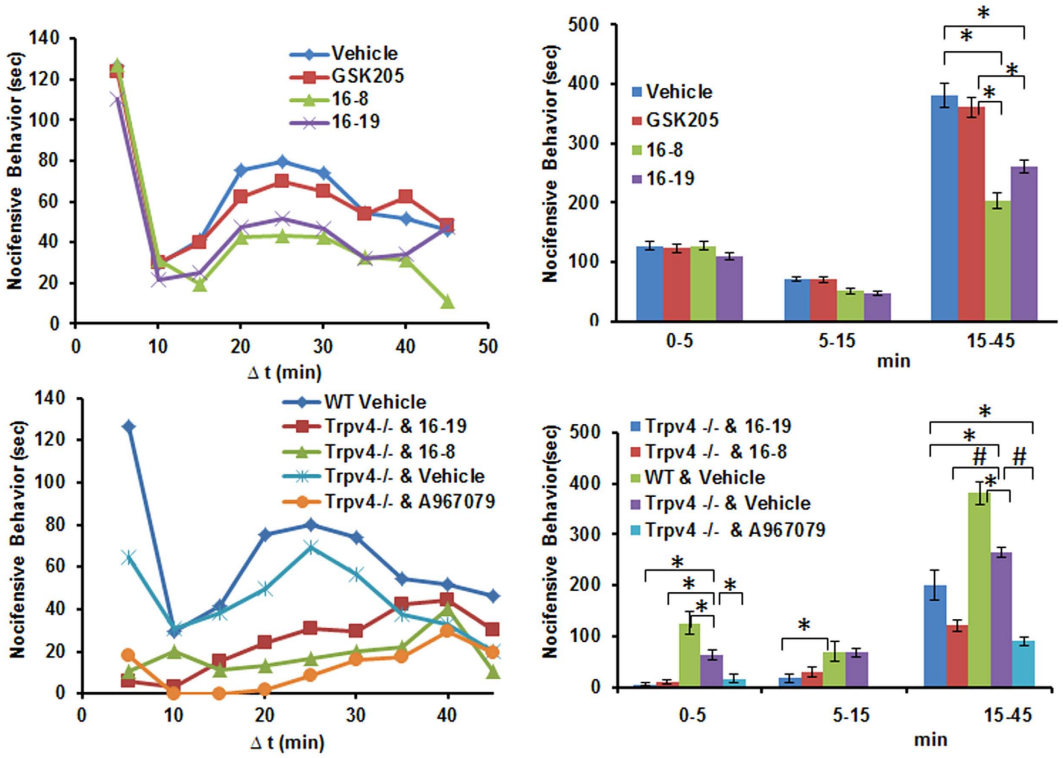

Figure 7. 16-8 and 16-19 effectively attenuate formalin-evoked trigeminal irritant pain. **(A)** Time-course of nocifensive behavior in WT mice following whisker-pad injection of 4% formalin. The mice were pre-injected (i.p., 10 mg/kg; 15 min before formalin) with GSK205, 16-8 or 16-19. Note effective reduction of nocifensive behavior in the late "neural" phase by compounds 16-8, 16-19, not by GSK205. **(B)** Cumulative response binned into 3 phases: acute phase (0–5 min), interphase (5–15 min), and late "neural" phase (15–45 min). Note significant reduction of nocifensive behavior in the late phase by 16-8, 16-19, not GSK205 ($^{*}P < 0.01$ vs vehicle and GSK205, one-way ANOVA). **(C)** As in (**A**), but also including *Trpv4*$^{-/-}$ mice. Compounds were applied i.p. 15 min before formalin challenge, at 10 mg/kg except established TRPA1 blocker, A967079 (25 mg/kg). Previously-established attenuated nocifensive behavior in early and late phase in *Trpv4*$^{-/-}$ mice was recapped, which was reduced further by TRPA1 blocker, A967079. **(D)** As in (**B**), plus inclusion of *Trpv4*$^{-/-}$ mice. Robust effects of TRPA1-blocker, A967079, were mimicked equi-potently by 16-8 and 16-19 for early phase, and by 16-8 for late phase, partially by 16-19 for late phase. (**A**,**C**) show averaged behavioral metrics per time-point, bars in (**B**,**D**) represent mean ± SEM; for (D) $^{*}P < 0.05$; $^{\#}P < 0.005$, one-way ANOVA; for all panels n = 5–8 mice/group.

translation potential due to unmet clinical need for new effective treatments in this condition[39], as well as the known role of both TRPV4 and TRPA1 in pancreatitis pain and inflammation[8,25,40].

Pancreatitis was induced with caerulein, a well-characterized model for acute pancreatitis[41]. Animals were treated with 10 mg/kg bw 16-8 by intraperitoneal injection, 30 min before induction of inflammation. We found significant attenuation of inflammatory parameters, namely edema, which was virtually eliminated in 16-8 treated animals. Furthermore, serum amylase, a marker of inflammatory injury of the pancreas, was significantly reduced by 16-8 treatment, as was myelo-peroxidase content of the pancreas, as a marker of inflammatory cell infiltration of the pancreas. The histopathological score for pancreas inflammation was also significantly reduced (Fig. 8A–E). Of note, pain behavior, similar to the effect of 16-8 on pancreas edema, was virtually eliminated upon treatment with compound 16-8. Thus, compound 16-8 was found to be highly effective in attenuating pain and inflammation of acute chemically-evoked pancreatitis.

Benign preliminary toxicity and pharmacokinetics of novel TPRV4/TRPA1 inhibitors. Promising properties of potent 16-… compounds prompted us to attempt to define their initial preliminary features in terms of *in-vivo* toxicity and pharmacokinetics, which will be followed by more extensive and in-depth investigations in future studies. For this, we chose compound 16-8 as the all-around most potent dual TRPV4/TRPA1 inhibitor, and also compound 16-19 with its potentially increased lipophilicity (Suppl Table 1). We measured compound concentration in several organs and plasma, and detected micromolar/submicromolar concentrations in liver and kidney, less than 100 nM concentrations in heart, brain, brainstem, trigeminal ganglion and skin. Of note, compounds were virtually undetectable in plasma (Suppl Fig. 1A). We detected higher concentrations of 16-19 in non-nervous tissue, especially liver and kidney, a pattern perhaps related to compound 16-19's increased lipophilicity. Based on this finding, we next examined 6 and 24 h time-points and detected 10-20 fold higher concentrations of 16-19 at the 6 h time-point, compared to the 1 h time-point, indicative of compound sequestration

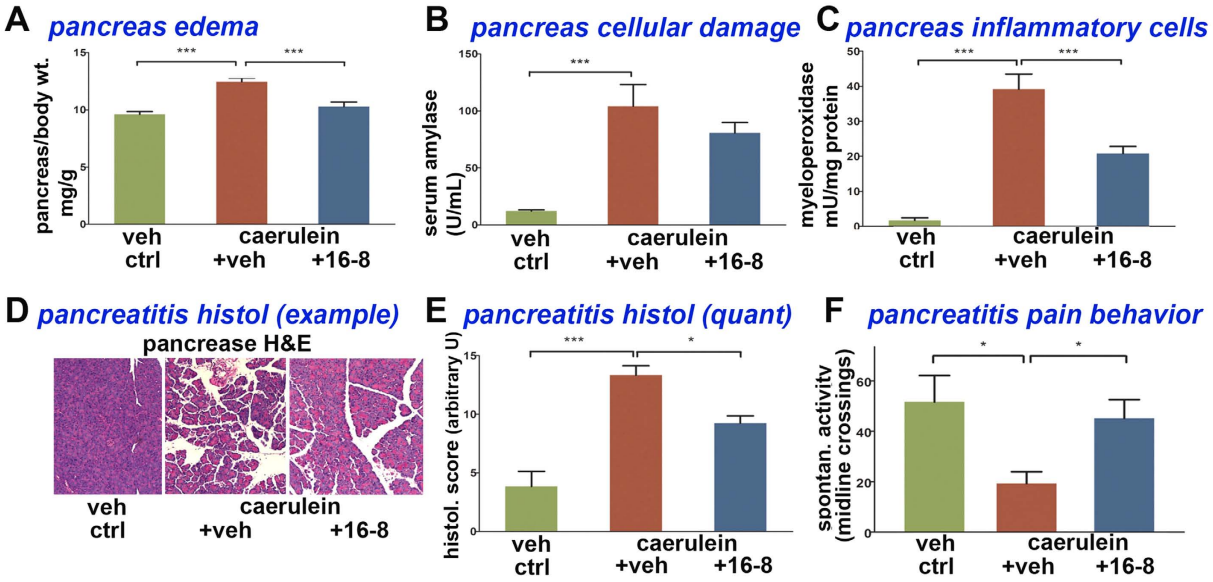

Figure 8. Compound 16-8 attenuates acute pancreatitis and improves pain behavior. (A) Caerulein-evoked acute pancreatitis causes pancreatic edema, which is eliminated by compound 16-8 (10 mg/kg, applied at 30 min before first exposure to caerulein). **(B)** Caerulein-evoked acute pancreatitis strongly elevates cellular toxicity marker amylase in serum. Amylase is reduced, but not significantly, in 16-8 treated animals. **(C)** caerulein-evoked acute pancreatitis causes elevated myelo-peroxidase (MPO) activity in serum, a marker for infiltration of inflammatory cells into the pancreas. MPO activity is significantly reduced in 16-8 treated mice. **(D)** caerulein-evoked acute pancreatitis can be readily demonstrated histologically, exemplified in the micrograph panels shown. Note increased pancreas inflammation in the middle-panel vs non-inflamed pancreas in vehicle-control challenged mice, and its attenuation by treatment with compound 16-8. **(E)** Bar diagram shows quantitation of inflammatory histologic parameters as shown in (**D**). Note significant increase of inflammation-index in caerulein acute pancreatitis mice, and its significant reduction upon treatment with compound 16-8. **(F)** Caerulein-evoked acute pancreatitis causes pain behavior, significantly reduced by compound 16-8. Note greatly reduced activity over the 6 h test period in caerulein-induced acute pancreatitis. This nocifensive behavior is greatly improved in response to systemic application of compound 16-8. Results are expressed as mean ± SEM; n = 6 mice/group; $^{*}P < 0.05$ (one-way ANOVA).

into solid organs (Suppl Fig. 1B–D). Values at the 24 h time-point were lower than at the 6 h but higher than at the 1 h time-point, indicating ongoing protracted compound clearance. We next confirmed that low/non-detectable concentrations in plasma were not caused by compound denaturation/degradation in plasma, as indicated in Suppl Fig. 1E, which shows no loss of detectable compound after 4 h incubation in plasma at 37 °C, conducted using compound 16-19.

We next performed basic preliminary *in-vivo* toxicity studies for compounds 16-8 and 16-19, both at 10 mg/kg bw, which was the effective concentration in both *in-vivo* pain models. We did not detect first evidence of cardiac, hepatic and renal toxicity, when comparing compounds 16-8 and 16-19 with vehicle (Suppl Fig. 2). For cardiac assessment, we did not detect differences and changes in heart rate over 1 h, conducted by EKG at the 6 h time-point. Serum creatinin as marker of renal function and alanine-amino-transferase (ALT) as marker of hepato-cellular integrity were not significantly elevated in animals treated with compounds 16-8 or 16-19. Thus, initial evidence for potent 16-… compounds highlights their acceptable preliminary pharmacokinetics properties as well as lack of gross systemic toxicity. Future studies will be necessary for more detailed assessment of the pharmacokinetics and toxicity of these compounds.

Discussion

Here we describe novel compounds that simultaneously inhibit both TRPV4 and TRPA1 ion channels. Both targets were inhibited by the novel "dual-inhibitors" at sub-micromolar potency in heterologous cellular channel activation assays. Furthermore, these compounds showed potent activity against TRPV4 in primary cells with native TRPV4 expression, and were more potent than their related parent-compound. The most potent compound identified here, compound 16-8, also showed a favorable activity profile in two pain-inflammation models, one of them a general irritant-pain model in the trigeminal system, the trigeminal formalin model, the other a visceral pain and inflammation assay with specificity for the pancreas, the caerulein-induced acute pancreatitis model. Of note, both *in-vivo* pain models have been shown previously to rely on co-contribution of TRPV4 and TRPA1. In this regard, several other relevant medical conditions, discussed in more detail below, also rely on TRPV4/TRPA1 and represent important unmet clinical needs that need to be addressed by translational-medical approaches. Therefore a potent "dual-inhibitor" for a specific combination of TRP ion channels, such as TRPV4 and TRPA1, could be highly beneficial in these indications.

More specifically, primary chondrocytes represent a cellular model for joint disease with involvement of TRPV4 in cartilage maintenance as well as arthritis/osteoarthritis. Recent evidence also suggests a potential role for TRPA1 in mediating joint pain in osteoarthritis[42]. In addition, TRPV4-expressing astrocytes are involved in many neurologic and psychiatric diseases such as pain, epilepsy, multiple sclerosis and other CNS autoimmune conditions, stroke, traumatic brain/spinal cord injury, brain edema, CNS infections, and more[35–37]. TRPA1 expression in astrocytes has also been reported[43]. Therefore, novel dual TRPV4/TRPA1 inhibitors might be suitable compounds for treatment of a number of diseases, from a spectrum of disorders affecting the nervous system as well as degenerative or inflammatory musculoskeletal conditions. For both types of disorders, we view compartmentalized application of compounds, i.e. intra-thecal or intra-articular delivery, as feasible future routes of delivery, in order to affect target cells more directly without affecting - TRPV4 and TRPA1 systemically.

In terms of *in-vivo* pain models, the trigeminal formalin model is rather a general model, not a direct pre-clinical translational-medical model. However, formalin models have a very robust track record in the identification of efficacious new compounds against pain[44]. Our findings with compound 16-8 in the trigeminal formalin model can be interpreted along these lines, especially for trigeminal pain including headaches[45,46]. In the context of these most prevalent neurological diseases, involvement of TRPV4 and TRPA1 has been reported previously in preclinical models, underscoring the case for their involvement with some compelling preclinical insights for both channels[47–50].

Pancreatitis in mice represents a more specific preclinical visceral inflammatory pain model, helpful to elucidate pathophysiology of this specific pain in order to better address a significant unmet medical need. In the current study pain and edema were strongly reduced by compound 16-8, the compound identified as showing the most advantageous features when tested in the trigeminal formalin pain model vs another high-potency dual-inhibitor, 16-19, and vs parent molecule, GSK205. This is in keeping with previous studies which demonstrated that both TRPA1 and TRPV4 contributed to pancreatic inflammation and pancreatic pain[25]. More precisely TRPA1 was shown to contribute to both pain and inflammation while TRPV4 contributed more selectively toward pain[8]. In our current study, there was significant attenuation of inflammatory parameters, but the reduction was not as robust as for pain and edema. This finding begets two important issues, namely (i) this could be a feature of the 2 targeted TRP channels, that they are more significant for pain than for inflammation (as concluded from experimental evidence in[14]), and (ii) edema of the pancreas, which is readily measurable by imaging techniques in patients, could possibly serve as a bio-marker for pancreatic pain. Beyond its role as biomarker, pancreas edema could sustain pancreas pain. At the pathophysiological level, edema will encompass edematous distension of the inflamed organ causing mechanical pain. This pain, amplified by inflammation of the painful organ, will in turn cause neurogenic inflammation, which will give rise to increased level of edema. This could evolve into a detrimental feed-forward mechanism. The specific effect of compound 16-8 on pancreatitis pain could be due to the compound interfering potently and efficiently with such a feed-forward mechanism as in (ii), by potently inhibiting both channels, as laid out in (i).

Beyond the two pain-inflammation conditions tested, TRPV4/TRPA1 co-involvement appears to play a role in several health-relevant conditions, such as colitis, itch, injury to airway and lungs via the inhalatory route and chronic cough[28,34,51–58]. In addition, interesting recent findings point toward a prominent role for TRPV4 in conditions as diverse as fibrotic disorders, UVB skin injury, and premature birth[15,59,60]. In these conditions, possibly via TRPA1-expressing innervating sensory neurons, a co-contribution by TRPA1 could be an important element. For such cases, 16-… compounds represent attractive candidates for effective treatment, to address the significant underlying unmet clinical need. Except pancreatitis, compound access to relevant target cells could also be readily accomplished by topical, non-systemic delivery via transdermal, transmucosal, inhalatory, intra-articular or intra-thecal formulations.

Our study presented here was strongly geared toward a translational medical agenda, meaning demonstration of effect of TRPV4/TRPA1 dual inhibitors, combined with a first-pass at pharmaco-tox assessment were our priority, rather than in-depth mechanistic studies. In addition, our goal was to demonstrate that modified 16-… compounds were more potent than the parent compound, GSK205. For future studies, in addition to continuation of a translational-medical agenda based on 16-… compounds, e.g. in-depth assessment of 16-… compounds at human isoforms of TRPV4 and TRPA1, effect of 16-… compounds in TRPV4/TRPA1-expressing primary human cells, our current results raise the following important questions/issues, namely a mandate to conduct mechanistic studies that address how TRPV4/TRPA1 dual inhibitors act on their respective target channels, and whether there is perhaps a shared mechanism between TRPV4 and TRPA1 of channel inhibition by potent 16-… compounds. In this context, it will be rewarding to zero in on a potential mechanism as to why compounds 16-8, 16-19 and to minor degree GSK205 are active against TRPA1 whereas compound 16-18 interestingly is not. Furthermore, the question why compound 16-19 fails to show increased potency over 16-8 will be an interesting one to address in future studies.

Materials and Methods

Compound synthesis. Compound synthesis is explicitly described in Supplementary Information (Suppl Fig. 3 and Suppl Tables 2–5).

Animals. 8–12 week old mice were used throughout the experiments. *Trpv4*$^{-/-}$ mice[3] have been outcrossed to WT (C57BL/6J) background and genotyped by PCR[3,15]. Animals were housed in climate-controlled rooms on a 12/12 h light/dark cycle with water and standardized rodent diet that was available ad libitum. All experiments were conducted in compliance and accordance with the guidelines of the NIH and the Institutional Animals' Care and Use Committee (IACUC) of Duke University, and under a valid IACUC protocol of the Duke University IACUC. All animal methods described in this publication were approved by the Duke University IACUC.

Trigeminal formalin irritant behavior – mouse model. Implementation of this model was conducted as described previously[13].

Video-taped nocifensive behavior was assessed by investigators blinded to genotype and treatment.

Acute pancreatitis mouse model. C57BL/6J male mice 8–10 weeks of age were subjected to acute pancreatitis by intraperitoneal injections of supramaximal doses of caerulein (50 μg/kg) every hour for a total of 6 h, as previously described in[61]. Control animals received 25% DMSO-saline solution by intraperitoneal injection every hour for 6 h. Compound 16-8 was dissolved in this vehicle (10 mg/kg) and injected i.p. 30 min prior to the first injection of caerulein. Animals were sacrificed 1 h after the last injection. Blood was collected and pancreatic tissue was promptly isolated, weighed for determination of pancreas wet weight/body weight ratio. Samples of tissues were fixed overnight in 10% neutral-buffered formalin, paraffin embedded and H&E-stained, or pancreatic tissue was quickly frozen and assessed for myeloperoxidase (MPO) activity. Serum amylase, MPO and histologic evaluation were conducted as described previously[61].

Assessment of nocifensive behavior[8]: Mice were housed in individual cages and video-recorded during the entire experiment. Two mice at a time were observed. Linear movement was measured as one event when mice passed through the median plane of the cage. Analysis began immediately after the first caerulein/vehicle injection and continued until the end of the experiment. Results were expressed as the sum of the movement events spanning the 6 h time-period following the first injection.

Cell cultures. N2a cells were used for directed expression of TRP ion channels as described previously[13]. TRPV4-eGFP from rat was used, previously found to respond to stimulation with GSK101 and hypotonicity in similar manner as native, non-fused TRPV4. All other channels were native channels from mouse, eGFP was co-transfected. Stimulation of over-expressed TRPV4 was conducted with GSK101 (5 nM), TRPV1 with capsaicin (10 μM), TRPV2 with hypotonicity (270 mosmol/L), TRPV3 with camphor (100 μM) and TRPA1 with mustard oil (100 μM). eGFP control-transfected N2a cells did not respond to these stimuli. Ca^{++} imaging was performed as described previously[13,14,34].

To visualize dose-response relationships, Hill plots were conducted using the Igor Pro software program, which derived the plots based on the following equation:

$$y = Base + (Max - Base) / \left\{ 1 + \left[\frac{x\ half}{x} \right]^{rate} \right\} \quad (1)$$

Primary porcine chondrocytes derived from femoral condyles of skeletally mature pigs were cultured and subjected to Ca^{++} imaging as described previously[19,32,62,63].

Astrocyte cultures were conducted following established protocols[64–66]. Astrocytes were prepared from Sprague Dawley rat embryos (E18). Briefly, the isolated cortices were minced, and then incubated with trypsin and DNase. Dissociated cells were suspended in Dulbeccco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively). Thereafter, cell suspensions were plated in 75 cm^2 tissue culture flasks (10×10^6 cells/flask) which were pre-coated with poly-L-lysine (10 μg/ml). The cells were maintained in a 10%CO_2 incubator at 37 °C. After 10–12 days, the media was removed and adherent cells were trypsinized (0.25%) and plated out onto coverslips for subsequent Ca^{++} imaging[34,67]. >95% of the cells were found to express astrocyte marker, glial fibrillary acidic protein (GFAP)[68].

Cell viability in culture. N2a cells were cultured in 96 well plates for 24–48 h. Cell viability studies relied on metabolic capability monitored with the indicator dye resazurin. Its reduction to resorufin (indicated by color change dark blue to pink) was monitored over time. Changes in absorbance at $\lambda = 570$ nm were recorded using a microplate reader (Molecular Devices). Metabolically active and viable cells shared the ability to reduce resazurin to resorufin whereas dead cells did not. Eight replicate cultures per experimental point were studied.

Assessment of hepatic, renal and cardiac function in mice treated with 16-… compounds. Mice were treated i.p. with compounds 16-8 and 16-19 (10 mg/kg). Hepatic and renal integrity were analyzed by alanine amino-transferase- and creatinine assays (Sigma), both relying on measurement of absorbance at $\lambda = 570$ nm in 96-well micro-titerplates. 8 technical replicates per animal were performed.

For heart rate assessment in mice treated with 16-8 and 16-19, animals were fitted with two electrodes, one to the ear, via clip, one to the rib-cage, using firm adhesive. Heart rate was monitored and analyzed using axoscope and clampfit 9.2 software (Molecular Devices)

Liquid Chromatography – Tandem Mass Spectrometry (LC-MS/MS). Mice were treated with 10 mg/kg i.p. of the respective inhibitor. Post-euthanasia harvested tissue was frozen in liquid nitrogen and stored at −80 °C for further analysis.

Frozen tissue samples were partially thawed and cut into 1 mm slices, 5–15 mg tissue, 2-fold excess water (mass/vol.), 6-fold excess acetonitrile (16-… compounds) or methanol (GSK205) containing appropriate amount of internal standard, and 2.5 mm zirconia/silica bead (Biospec Products Inc.) were added to 500-μL polypropylene (PP) conical tube, homogenized in a Fast-Prep apparatus (Thermo-Savant) at speed "4" for 20 sec at room temp, and centrifuged at 13,600 g for 5 min at room temp. Depending on the expected concentration range of the measured compound, the supernatant was diluted 1/4–1/20 (in Mobile phase A, see below) and placed in autosampler for LC-MS/MS analysis.

The LC-MS/MS assay for 16-… compounds and GSK205 was developed on an Agilent 1200 series LC system interfaced with Applied Biosystems API 5500QTrap, a hybrid triple quadruple-linear trap MS/MS spectrometer.

Analyst (version 1.6.1) software was used for mass parameters tuning, data acquisition, and quantification. LC column: 3 × 4 mm RP C18 (Phenomenex, AJ0-4287) was operated at 35 °C. Mobile phase A: 0.1% formic acid, 2% acetonitrile, in LC/MS-grade water; mobile phase B: acetonitrile; flow rate: 1 mL/min, 1:1 MS/MS:waste split. Run time was 4 min. Diverter valve was used to send flow to MS/MS only between 1.2 and 2.5 min. The elution gradient was: 0–0.5 min, 1%B; 0.5–1.2 min, 1–95%B; 1.2–1.5 min, 95%B; 1.5–1.6 min, 95–1%B. Autosampler was operated at 4 °C; injection volume was kept at 10–50 μL. Electrospray ionization (ESI) source parameters were: positive ionization mode, curtain gas flow = 30, ionization potential = 5500 V, temperature = 500 °C, nebulizing gas 1 flow = 30, nebulizing gas 2 = 30, declustering potential = 20 V. 16-… compounds and GSK205 were individually infused as 100 nM solutions in 50%A/50%B at 10 μL/min flow rate and parameters optimized to provide maximal ion count for "parent" and collision-produced ("daughter") MS/MS ions. Parent/daughter quantifier [qualifier] ions utilized: GSK205 (401.1/280[370]), 16-8 (400.1/279.1[91.1], 16-16 (387.1/280[105]), 16-18 (415.2/280[370], 16-19 (414.1/279.2[91.1]. Standard (analyte of interest)/internal standard pairs utilized: GSK205/16-16, 16-8/16-16, 16-18/GSK205, 16-19/16-8.

Calibration samples (n = 6) were prepared by adding pure standard of the measured compound to tissue homogenate (tissue + 2-fold excess water, mass/vol) in the appropriate range needed for the particular dosing regime. Organs studied were analyzed alongside the study samples. The following are typical ranges used (the lower value representing also the LLOQ at 80% accuracy limit, all other calibrator levels at 85% accuracy limit): 0.38–6 nM (plasma), 6–100 nM (skin), 6–48 nM (heart), 7.5–120 nM (brain), 19–300 or 1500–24000 nM (liver), 56–900 or 1500–12000 nM (kidney), 500–8000 nM (fat). Peak integration, calibration, and quantification was performed within Analyst software. The response of the peak area standard/int. std. to nominal concentration was linear with r = 0.999 or better.

Patch Clamp Recordings. Heterologously transfected N2a cells were subjected to patch clamp electrophysiological recordings. Briefly, 24 h after transfection cells were prewashed with extracellular fluid (ECF) which contained (in mM) 1 $MgCl_2$, 10 Glucose, 10 HEPES, 145 NaCl and 2 $CaCl_2$ (pH 7.4, 310mOsM). Cells were then incubated with or without TRPV4 inhibitors in ECF for 5 min before whole cell recording. Cover slips were transferred to a recording chamber mounted on the stage of a Leica inverted microscope that was equipped with fluorescent filters. Transfected cells were identified before patching by their green florescent color. Cells were patched with a 2.5–3.0 MΩ glass electrode pulled from borosilicate glass capillaries using pipette puller (Sutter instruments). The intracellular solution contained (mM) 140 CsCl, 10 HEPES, 1 EGTA, 0.3 Na-GTP, 2 Na_2-ATP, and 2 $MgCl_2$ (pH 7.4, 295 mOsm). Whole cell currents were recorded using pclamp 9.2 software and Axopatch 200B amplifier (Molecular Devices). The cells were first clamped at −65 mV before applying a 1 s voltage ramp from −110 mV to +120 mV. The voltage ramp was applied every 2 seconds for 15 to 20 sweeps. Capacitance was monitored throughout the experimental recordings. Reported data was within ±3 pF.

Statistical Analysis. Data are expressed as mean ± SEM. Two-tail *t*-tests or one-way ANOVA followed by Tukey *post-hoc* test were used for group comparisons. $P < 0.05$ indicated statistically significant differences

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Author Contributions

P.K. conducted experiments, analyzed data, wrote paper, conceptual input. Y.C. conducted experiments, analyzed data. W.L. conducted experiments, analyzed data. M.Y. conducted experiments, analyzed data, wrote paper. S.H.L. conducted experiments. J.R. conducted experiments, analyzed data. R.S. conducted experiments, analyzed data. P.F. conducted experiments, analyzed data. D.M.G. conducted experiments, analyzed data. S.A.S. wrote paper, conceptual input. I.S. conducted experiments, conceptual input. R.A.M. wrote paper, conceptual input, analyzed data. R.A.L. wrote paper, conceptual input, analyzed data. F.G. wrote paper, conceptual input, analyzed data. W.B.L. wrote paper, conceptual input, analyzed data.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: Content as reported in this paper has been included in patent applications by the Duke University Office of Licensing and Ventures.

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Supplementary File

Title:

Small molecule dual-inhibitors of TRPV4 and TRPA1 for attenuation of inflammation and pain

Authors:

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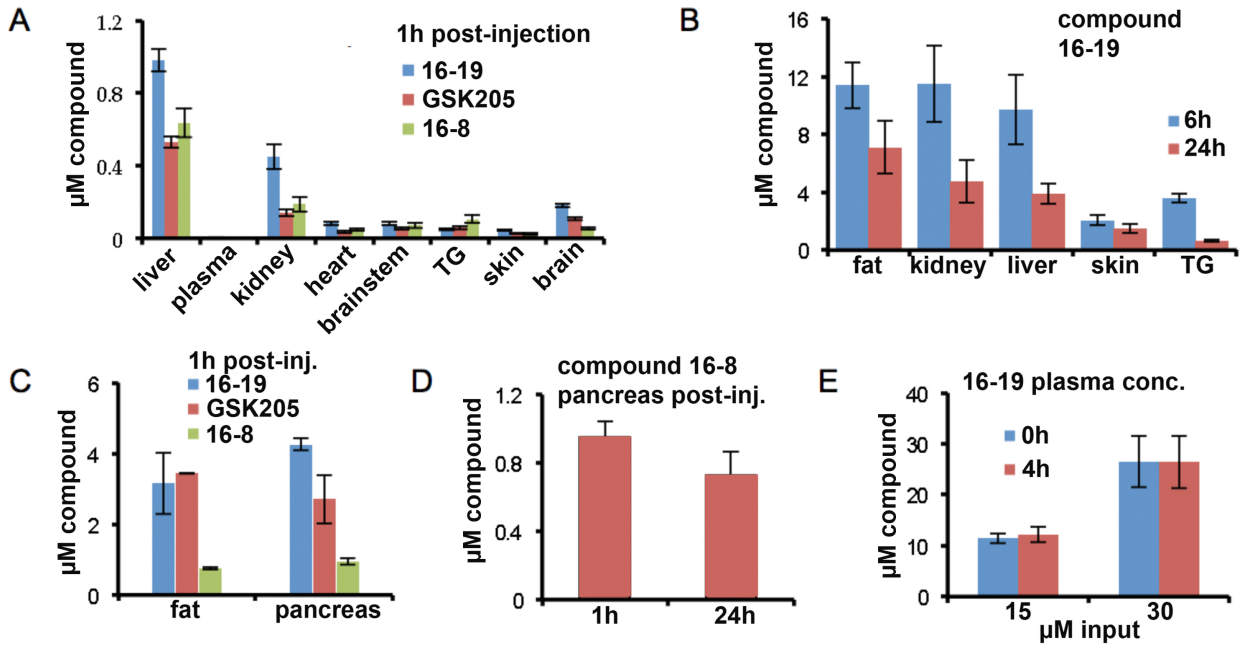
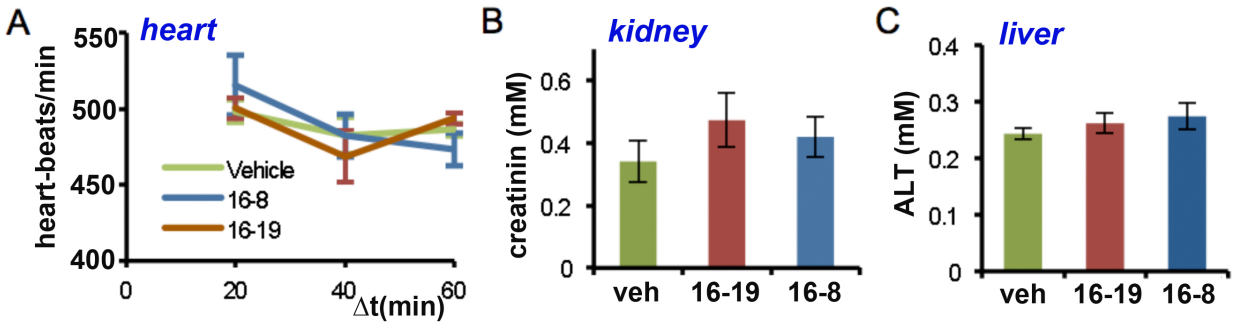


Suppl Fig. 1: *Pharmaco-kinetics/pharmaco-tox of compounds 16-8, 16-19 and GSK205 in-vivo*

(A) concentrations of compounds 16-8, 16-19 and GSK205 (10mg/kg) in several murine tissues/organs 1h post-i.p. injection. **(B)** Compound 16-19 time-course at 6h and 24h in several organs. 16-19 was selected because of its elevated levels at the 1h time-point, and based on the estimate that 16-19 is more lipophilic than 16-8 and GSK205. Note that metrics at 6h are invariably higher than at 24h. All values are appreciably higher than at 1h. **(C)** Concentrations of 16-8, 16-19 and GSK205 in fat and pancreas after one hour. Note lower concentration of 16-8 vs. 16-19 and GSK205, yet above its IC50. **(D)** Concentrations of 16-8 in the pancreas at 1h and 24h time-points. **(E)** Structural stability of compound 16-19 in plasma as suggested by stable concentration after 4h/37˚C. Results are expressed as means±SEM, n=6 mice/experimental group for all expts.



Suppl Fig. 2: *Absence of cardiac, renal and hepatic toxicty of compounds 16-8, 16-19*
(A) Heart rate time-course after i.p. injection (10mg/kg) of compounds. There was no significant difference in heart rates between vehicle and compounds. **(B)** Serum creatinin was not significantly elevated in animals treated with 16-19 and 16-8 vs vehicle control. **(C)** Serum alanin-amino-transferase (ALT) levels were not significantly elevated in animals treated with 16-19 and 16-8 vs vehicle control. n=6 mice/group for all expts.

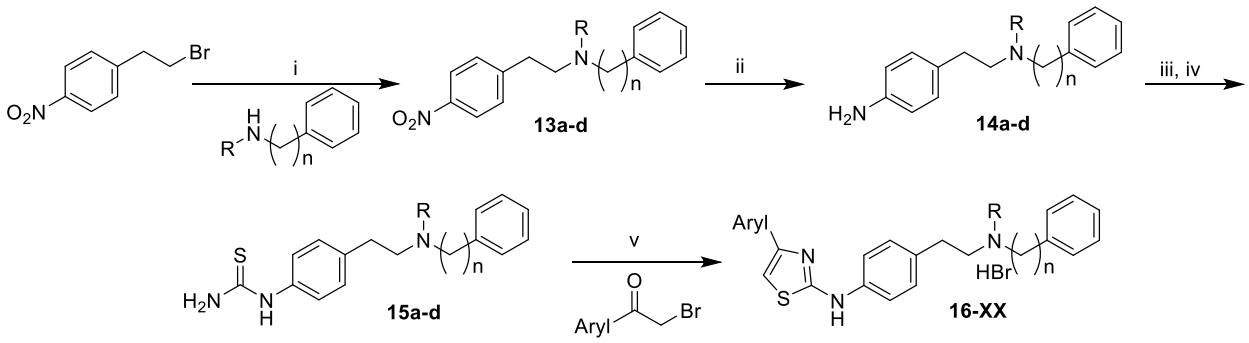
Suppl Table 1

Properties GSK205, 16-8 and 16-19

Compound	MW	PSA	c-logP(oct/H_2O)
GSK205	400	37.53	5.35
16-8	399.6	24.4	6.33
16-19	413.6	24.2	6.58

MW – molecular weight
PSA – polar surface area
c-logP(oct/H_2O) indicates: calculated logP(octanol solubility / H_2O solubility)

Suppl Fig 3 General synthetic scheme for compounds 16-08 to 16-19.



Reagents and conditions: (i) K_2CO_3, CH_3CN. (ii) Zn, MeOH, 12M HCl. (iii) 1,1′-Thiocarbonyldiimidazole. (iv) 7M NH_3 in MeOH. (v) EtOH, reflux

General procedure for the S_N2 displacement of 4-nitrophenethyl bromide:
Powdered, oven-dried K_2CO_3 (1.5 eq.) and the amine (1.5 eq.) were added sequentially to a room temperature solution of the bromide (0.33 M) in anhydrous CH_3CN. The reaction mixture was heated to 80 ^oC (oil bath temp) until analysis of the reaction mixture by LCMS indicated complete consumption of the bromide (~6-18h). The mixture was cooled to room temperature and diluted with brine (two volume equivalents). The resulting emulsion was extracted with EtOAc (2 x one volume equivalent). The combined extracts were added to silica gel (mass of silica gel = 2x mass of starting bromide) and the mixture was concentrated to dryness under reduced pressure. Flash column chromatography (RediSepR_f SiO_2, 100% CH_2Cl_2→ 5% MeOH in CH_2Cl_2) gave the product as a brown to amber oil.

Suppl Table 2. Yield of tertiary amines 13a-d

Entry	Compound number	R	n	yield
1	13a	Me	0	17%
2	13b	Me	1	49%
3	13c	Me	2	42%
4	13d	Et	1	15%

General Procedure for the nitro to aniline reduction: A solution of the nitro compound (0.5 M in MeOH) was cooled in an ice-NaCl bath. Zinc dust (4.5 eq.) was added in one portion followed by drop wise addition of 12M HCl (4.5 eq.) over 2-3 minutes. After 1h, the cooling bath was removed and the reaction mixture was allowed to stir over night at room temperature. The following morning, the mixture was cooled in an ice-NaCl bath once again and 30% aqueous NaOH was added drop wise until pH 14 (universal indicating pH paper) was reached. The mixture was diluted with CH_2Cl_2 (five volume equivalents) and stirred for 5 minutes. After this time, insolubles were removed at the vacuum and the filter cake was washed with CH_2Cl_2 (2 x 25 mL). The organic phase of the filtrate was separated, washed with brine (100 mL) and dried ($MgSO_4$). The drying agent was removed by filtration. Silica gel (~5g) was added and the filtrate was concentrated to dryness under reduced pressure. Flash column chromatography (RediSepR_f SiO_2, 100% CH_2Cl_2→ 5% MeOH in CH_2Cl_2) gave the product as a clear, amber oil.

Suppl Table 3. Yield of anilines 14a-d

Entry	Compound number	R	n	yield
1	14a	Me	0	75%
2	14b	Me	1	84%
3	14c	Me	2	97%
4	14d	Et	1	85%

General procedure for thiourea formation: A solution of the aniline (0.22 M) in anhydrous CH_2Cl_2 was added drop wise over 2-5 minutes to an ice-NaCl bath cooled solution of 1,1'-thiocarbonyldiimidazole (2 eq., 0.15 M) in anhydrous CH_2Cl_2. After 15 minutes, the cooling bath was removed and the reaction mixture was stirred at room temperature until analysis by TLC (5% MeOH in CH_2Cl_2) indicated complete consumption of the starting aniline. The mixture was cooled once again in an ice bath and 7M NH_3 in MeOH (10.5 eq.) was added drop wise over 2-5 minutes. The bath was removed and the mixture was stirred over night at room temperature. Silica gel (mass of silica gel = 2x mass of starting aniline) was added and the mixture was concentrated to dryness under reduce pressure. Flash column chromatography (RediSepR_f SiO_2, 100% CH_2Cl_2 → 10% MeOH in CH_2Cl_2) gave the pure thiourea.

Suppl Table 4. Yield of thioureas 15a-d

Entry	Compound number	R	n	yield
1	15a	Me	0	99%
2	15b	Me	1	96%
3	15c	Me	2	88%
4	15d	Et	1	67%

General procedure for thiazole formation: A mixture of the thiourea (0.1 M) in EtOH and the α-bromoacetophenone derivative (1.1 eq.) was heated to 75 oC (oil bath temperature) until analysis by TLC (5% MeOH in CH_2Cl_2) indicated complete consumption of the thiourea. Silica gel (mass of silica gel = 2x mass of starting thiourea) was added and the mixture was concentrated to dryness under reduce pressure. Flash column chromatography (RediSepRf SiO_2, 100% CH_2Cl_2 → 10% MeOH in CH_2Cl_2) gave the pure thiazole hydrobromide**.**

Suppl Table 5. Yield of thiazole hydrobromides 16-08 to 16-19

Entry	Compound number	R	n	aryl	yield
1	16-08	Me	1	phenyl	56%
2	16-12	Me	2	3-pyridyl	82%
3	16-13	Me	1	4-pyridyl	83%
4	16-14	Me	1	2-pyridyl	94%
5	16-16	Me	0	3-pyridyl	98%
6	16-18	Et	1	3-pyridyl	31%
7	16-19	Et	1	phenyl	93%
8	16-43C	Me	1	3-pyridyl	52%

⌘ *Author's Choice*

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Transient Receptor Potential Vanilloid 4 Ion Channel Functions as a Pruriceptor in Epidermal Keratinocytes to Evoke Histaminergic Itch*

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TRPV4 ion channels function in epidermal keratinocytes and in innervating sensory neurons; however, the contribution of the channel in either cell to neurosensory function remains to be elucidated. We recently reported TRPV4 as a critical component of the keratinocyte machinery that responds to ultraviolet B (UVB) and functions critically to convert the keratinocyte into a pain-generator cell after excess UVB exposure. One key mechanism in keratinocytes was increased expression and secretion of endothelin-1, which is also a known pruritogen. Here we address the question of whether TRPV4 in skin keratinocytes functions in itch, as a particular form of "forefront" signaling in non-neural cells. Our results support this novel concept based on attenuated scratching behavior in response to histaminergic (histamine, compound 48/80, endothelin-1), not non-histaminergic (chloroquine) pruritogens in *Trpv4* keratinocyte-specific and inducible knock-out mice. We demonstrate that keratinocytes rely on TRPV4 for calcium influx in response to histaminergic pruritogens. TRPV4 activation in keratinocytes evokes phosphorylation of mitogen-activated protein kinase, ERK, for histaminergic pruritogens. This finding is relevant because we observed robust anti-pruritic effects with topical applications of selective inhibitors for TRPV4 and also for MEK, the kinase upstream of ERK, suggesting that calcium influx via TRPV4 in keratinocytes leads to ERK-phosphorylation, which in turn rapidly converts the keratinocyte into an organismal itch-generator cell. In support of this concept we found that scratching behavior, evoked by direct intradermal activation of TRPV4, was critically dependent on TRPV4 expression in keratinocytes. Thus, TRPV4 functions as a pruriceptor-TRP in skin keratinocytes in histaminergic itch, a novel basic concept with translational-medical relevance.

Itch is a clinical problem that leaves many sufferers insufficiently treated, with >20 million in the United States (1–3). This is also caused by incomplete understanding of its molecular, cellular, and cell-to-cell signaling mechanisms. Neural pathways have been understood as key for itch, whereby specialized primary sensory pruriceptor neurons relay sensory afferent information to itch-transmitting neural pathways, ultimately evoking the sensation of itch (2, 4–8). Exogenous or endogenous pruritogens are thought to act on primary sensory neurons, producing the sensation of itch by activating the pruriceptors expressed by these afferents. Primary pruriceptor neurons may receive modulatory signals from atopic inflammatory cells, such as mast cells, and also from epidermal keratinocytes (7). It was recently elucidated that the atopia cytokine, thymic stromal lymphopoietin (TSLP), was secreted from skin keratinocytes to activate TRPA1 ion channels on primary pruriceptor neurons and induced itch (9). Despite this landmark discovery, mechanisms of how the epidermal keratinocyte specifically functions to evoke itch remain largely unknown, especially mechanistic insights that rely on precise genetic targeting of genes-of-interest only in keratinocytes. In other words, molecular and cell-to-cell signaling mechanisms of forefront pruri-transduction are elusive.

We recently defined a mechanism of how ultraviolet B (UVB)[3] radiation activates TRPV4 ion channels in skin epidermal keratinocytes (10). Their genetically encoded, inducible absence in skin keratinocytes suffices to contain pain and tissue damage evoked by UVB overexposure. In skin keratinocytes, TRPV4 activation by UVB is potentiated by endothelin-1 (ET-1) via endothelin receptors A and B. TRPV4-activation in these cells leads to Ca^{2+} influx, which in turn increases gene expression of ET-1, providing the substrate of a feed-forward mechanism that sustains organismal pain. This is an interesting observation in the context of itch because ET-1 injection into skin is known to cause itch in human subjects and evokes scratching behavior in experimental animals upon intradermal

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[3] The abbreviations used are: UVB, ultraviolet B; TRPV4, transient receptor potential (TRP) vanilloid 4; ET-1, endothelin-1; tam, tamoxifen.

injection (11–16). TRPV4 has been implicated in other forms of pain (10, 17–26). It is a multimodally activated TRPV channel, *e.g.* activated by changes in osmotic pressure, mechanical, UVB, and chemical cues and modified by thermal cues (27–31). Except for the recent elucidation of the role of TRPV4 as ionotropic receptor for UVB in keratinocytes to reprogram these cells into organismal pain generators, its role in pain has been attributed to its expression in primary sensory neurons.

Against this background, especially the finding of TRPV4-dependent secretion of the pruritogen, ET-1, by keratinocytes, we felt that we have raised a timely question, namely whether TRPV4 plays a role in itch, in particular whether TRPV4 in keratinocytes of the epidermis can drive scratching behavior. To address this question we decided to first focus on acute itch and, specifically, as an initial priority, to examine prototypic examples of histaminergic itch, including ET-1-evoked itch, plus chloroquine-caused non-histaminergic itch. In this study we are reporting an exciting new function of TRPV4 in forefront signaling of the integument, namely that TRPV4 in epidermal keratinocytes functions as a pruriceptor-TRP channel in acute histaminergic itch, including itch evoked by ET-1, not in non-histaminergic itch evoked by chloroquine. Direct activation of TRPV4 channels also evokes scratching behavior, which appears completely dependent on TRPV4 expression in keratinocytes, thus underscoring the role of this cell and its expression of TRPV4 in itch. Complementing findings in our *Trpv4* keratinocyte-specific inducible knock-out (*Trpv4* cKO) mice, we demonstrate Ca^{2+} transients in response to histaminergic pruritogens in cultured primary keratinocytes that depend on TRPV4. Ca^{2+} influx via TRPV4 then up-regulates phosphorylation of the mitogen-activated protein kinase ERK in keratinocytes. Consequently, we find topical transdermal treatment with a selective inhibitor of TRPV4 to function efficiently as an anti-pruritogen. Moreover, we observed similar *in vivo* anti-pruritic effects when topically targeting MEK, upstream of ERK, with a selective inhibitor.

Experimental Procedures

Animals—The pan-null phenotype of *Trpv4*$^{-/-}$ mice relies on excision of the exon encoding transmembrane domains 5–6. Mice were outcrossed to C57BL/6J background and PCR-genotyped (10, 25, 26, 32). Male WT (C57BL/6J) and *Trpv4*$^{-/-}$, 2–2.5 months of age, were used for all experiments.

Keratinocyte-specific, tamoxifen (tam)-inducible *Trpv4* knockdown mice were used as previously described (10). In brief, the *Trpv4* genomic locus was engineered so that loxP sites surrounded exon 13, which encodes TM5–6. This mutation was propagated in mice that were crossed to K14-CRE-ER^{tam} mice, so that *Trpv4*$^{lox/lox}$x(K14-CRE-ER^{tam}) mice could be induced by tam (Sigma) administration via oral gavage for five consecutive days at 6 mg/day in 0.3 ml corn oil at age 2–2.5 months of age, plus a 1-time booster 2 weeks after the last application. Control animals received the same volume of corn oil. Efficiency of targeting was verified by quantitative real-time PCR and immunohistochemistry for *Trpv4* expression in skin at gene and protein levels, respectively (10). Both male and female mice were used for *in vivo* scratching behavior as shown in Figs. 1 and 5, and no difference was detected between sexes.

Animals were housed in climate-controlled rooms on a 12/12-h light/dark cycle with water and a standardized rodent diet available *ad libitum*. All animal protocols were approved by the Duke University Institutional Animal Care and Use Committee in compliance with National Institutes of Health guidelines.

Drugs—Histamine, compound 48/80, endothelin-1, chloroquine, and GSK1016790A (GSK101) were purchased from Sigma. GSK2193874 (GSK219) was obtained from Tocris, U0126 was from Selleckchem, and GSK205 was synthesized (26, 33). All were dissolved in sterile normal saline except that GSK101 and GSK205 were dissolved in DMSO (20 mM in stock) and further diluted until use.

Topical Treatment Formulation—Compounds GSK205 and U0126 were kept as DMSO stock, then diluted to 100 μM and 0.1 mg/ml, respectively, in 25% isopropyl alcohol, 15% ethanol, and 60% glycerol when used.

Itch Behavioral Tests—Mice were shaved at the dorsal neck where intradermal injections and topical applications were applied. Mice were allowed to acclimate to a Plexiglas chamber for at least 30 min before testing and received intradermal injection of pruritogens (histamine, 500 μg/50 μl; 48/80, 100 μg/50 μl; ET-1, 25 ng/50 μl; chloroquine, 200 μg/50 μl) or saline through a 30-gauge needle into the nape of neck to elicit scratching behavior. After injection, mice were immediately placed back in the chamber, and the scratching behavior was recorded by a Panasonic video camera for a 30-min observation period. Hind limb scratching behavior directed toward the shaved area at the nape of neck was observed. One scratch is defined as a lifting of the hind limb toward the injection site and then a replacing of the limb back to the floor, regardless of how many scratching strokes take place between those two movements. Behavioral analysis was conducted by observers blinded to genotype.

To investigate the topical effects of the specific TRPV4 inhibitor GSK205 or the specific MEK inhibitor U0126 on pruritogen-induced scratching behaviors, mice received a transdermal-topical application of 100 μl of formulated GSK205 (100 μM) or U0126 (0.1 mg/ml) on the shaved area at the nape of neck 20 min before pruritogen injections. Control animals received the same volume of placebo.

Keratinocytes Culture and Ca^{2+} Imaging—Primary mouse keratinocytes were cultured following previous protocol (10). The epidermis from the back skin of newborn WT mice (P0-P2) was separated from the dermis by floating the skin on 0.25% trypsin (Gibco) for 14–18 h at 4 °C. Basal keratinocytes were separated from the cornified sheets by filtration through a 70 μM cell strainer (BD Biosciences). Keratinocytes were plated on collagen-coated dishes or glass coverslips and grown in EME media (Gibco) supplemented with bovine pituitary extract and epidermal growth factor, 10% chelexed fetal bovine serum (Gibco), 100 pmol of cholera toxin (Calbiochem), and 1× antibiotics/antimycotics (Gibco) in an incubator at 5% CO_2 and 37 °C.

Primary human keratinocytes were cultured as previously described (34). In brief, surgically discarded foreskin samples, obtained from Duke Children's Hospital in accordance to institutionally approved IRB protocol, were incubated with Dispase

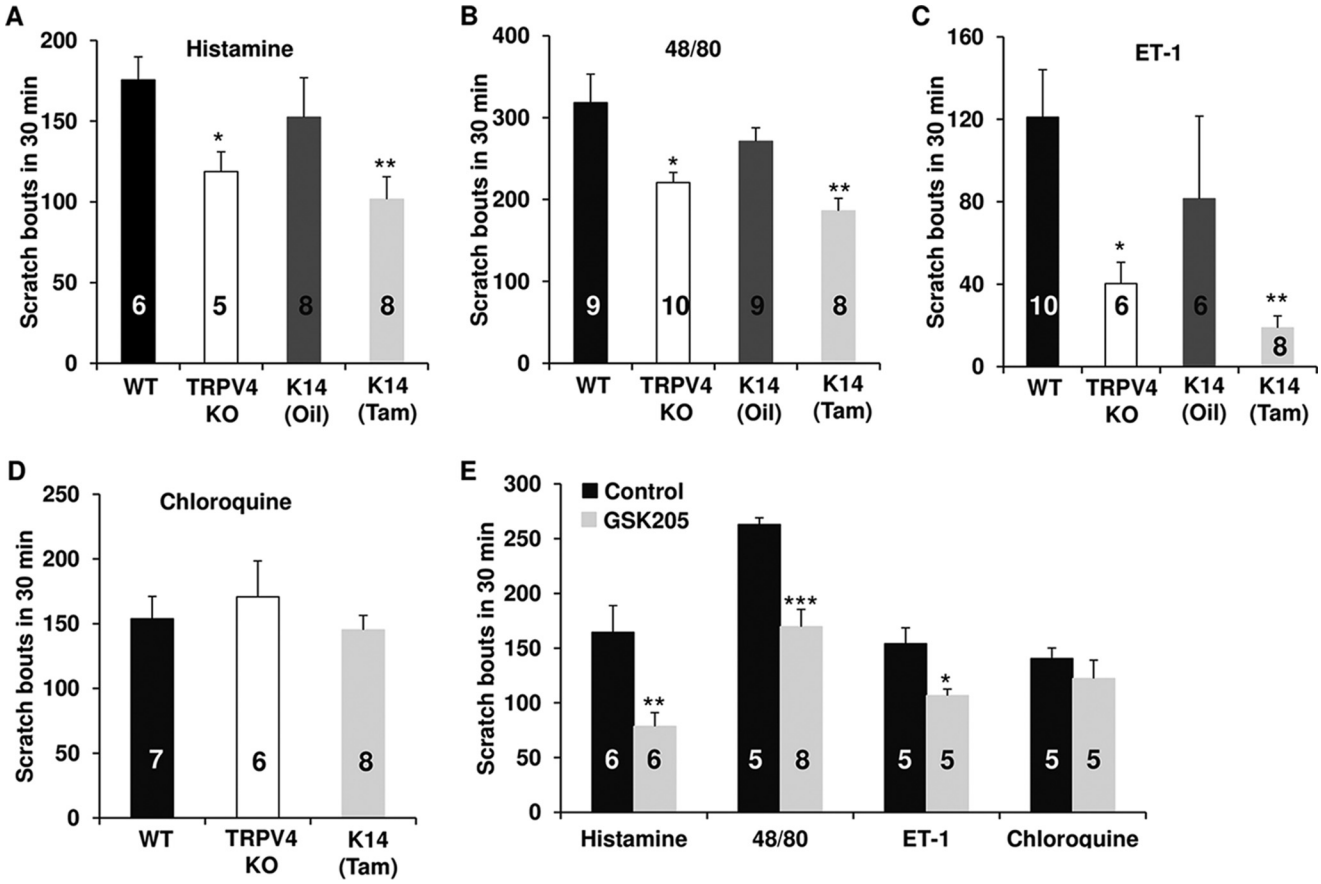

FIGURE 1. ***Trpv4* in skin keratinocytes is essential for histamine-dependent itch.** Histamine (*A*), compound 48/80 (*B*), and ET-1 (*C*), but not chloroquine (*D*), evoked acute scratching behaviors that were significantly attenuated in *Trpv4* cKO (K14-Tam) and pan-null mice (TRPV4 KO) *versus* their respective controls (*A–D*, *, $p < 0.05$; **, $p < 0.01$ *versus* WT). Mice topically transdermally treated with the TRPV4-selective inhibitor GSK205 showed a significant reduction of scratching behaviors (*E*, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ *versus Control*). One-way analysis of variance with Tukey's post hoc test was used for *A–D*, and two-tail *t* test was used for *E*. Group size is indicated in the *bars*.

(Gibco, 4 units/ml) for 12–16 h at 4 °C followed by 0.05% trypsin (Gibco) for 10–20 min at 37 °C. Cells were grown and passaged in keratinocyte serum-free media (Invitrogen) at 37 °C with 5% CO_2 and used at passage 2–3.

Ca^{2+} imaging of primary epidermal keratinocytes in response to chemicals was conducted after loading with 2 μM fura2-AM (Invitrogen) for 30 min after a ratiometric Ca^{2+}-imaging protocol with 340/380-nm blue light for dual excitation. Ratios of emissions were acquired at 0.5 Hz. $\Delta R/R_0$ was determined as the fraction of the increase of a given ratio over baseline ratio divided by baseline ratio.

To investigate the effects of the specific TRPV4 inhibitors GSK205 or GSK219 on pruritogen-induced Ca^{2+} influx and pERK expression, cells were incubated with GSK205 or GSK219 for 15 min before stimulation. Control cells received the same volume of vehicle.

Western Blot—Routine procedures were followed (10, 25, 26, 35). Briefly, cultured keratinocytes and dissected dorsal skin (0.5 × 0.5 cm, the area that received the treatment) were protein-extracted in radioimmunoprecipitation assay (RIPA, Sigma) buffer and electroblotted to nitrocellulose membranes after gel separation of proteins in a 4–15% polyacrylamide gel (Bio-Rad). Membranes were blocked with 5% BSA (Sigma) in TBST, and pERK and ERK were specifically detected with primary antibodies (rabbit anti-pERK (catalog #9101) and anti-ERK (catalog #4695), both at 1:2000; Cell Signaling Technology), secondary antibody (anti-rabbit peroxidase-conjugated, 1:5000; Jackson ImmunoResearch), and chemiluminescence substrate (ECL-Advance, GE Healthcare). Abundance was quantified using ImagePro Plus software. β-Actin, as a control, was detected with a mouse monoclonal anti-β-actin antibody (1:4000; catalog #sc-47778, Santa Cruz) or a rabbit polyclonal anti-β-actin antibody (1:4000; catalog #A5316, Sigma). Immunoblot band intensity was quantitated using the software Image J (National Institutes of Health).

Statistical Analysis—All data are expressed as the mean ± S.E. Two-tailed *t* tests or one-way analysis of variance followed by Tukey's post hoc test were used for group comparisons. $p <$ 0.05 indicated statistically significant differences.

Results

Trpv4 in Skin Keratinocyte Is Critical for Histaminergic Itch—To assess the contribution of keratinocyte TRPV4 channels to acute itch, we subjected *Trpv4* cKO mice to intradermal injections of both histaminergic and non-histaminergic pruritogens. Throughout, we also challenged *Trpv4* pan-null mice in order to be able to compare any eventual behavioral phenotype present in *Trpv4* cKO mice with that in the respective pan-null mouse. All histaminergic pruritogens including ET-1 evoked a solid scratching response, namely histamine itself (Fig. 1*A*), the

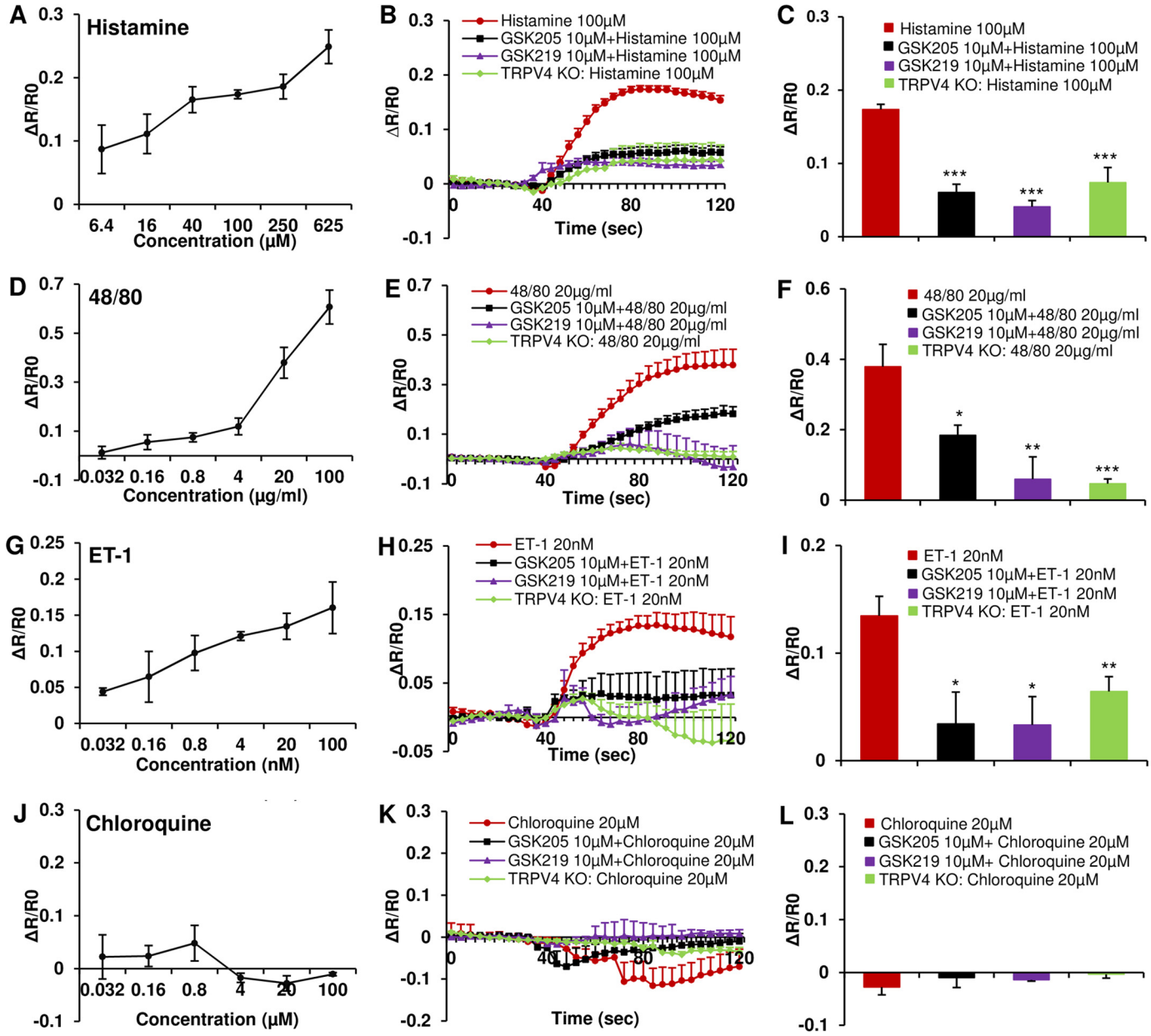

FIGURE 2. **Histamine-dependent pruritogens evoke Ca^{2+} influx in cultured keratinocytes via TRPV4 channels.** Histamine-dependent pruritogens evoke Ca^{2+} influx in cultured keratinocytes via TRPV4 channels. Histamine (*A*), compound 48/80 (*D*), and the partial histaminergic ET-1 (*G*), but not chloroquine (*J*), triggered Ca^{2+} influx in a dose-dependent manner in keratinocytes. The evoked $Ca2^{2+}$ signal was attenuated in cells pretreated with GSK205 or GSK219, both TRPV4-selective inhibitors, and also in cells from *Trpv4*$^{-/-}$ mice (*B* and *C*, *E* and *F*, *H* and *I*, and *K* and *L*; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ *versus* the respective pruritogens). Two-tail *t* test was used for statistic analyses. $n = 150–300$ cells/treatment.

polymeric secretagogue and MrgprX2 activator, compound 48/80 (36–40) (Fig. 1*B*), and the partial histaminergic ET-1 (13, 16, 40–43) (Fig. 1*C*), as did the non-histaminergic chloroquine (40, 44–46) (Fig. 1*D*). The scratching responses evoked by histaminergic pruritogens were significantly attenuated in *Trpv4* cKO mice, most robustly for ET-1. In contrast, scratching in response to chloroquine was not (Fig. 1, *A*–*D*). This means that TRPV4 ion channels in skin keratinocytes powerfully control organismal itch-related scratching behavior by converting the epidermal keratinocyte into an itch-generator cell that directly or indirectly signals to peripheral pruriceptor sensory neurons. In keeping with this new basic concept, *Trpv4* pan-null mice had a similar profile (Fig. 1, *A*–*D*), their reduced scratching in response to histaminergic pruritogens, indicating that genetically encoded pan-organismal absence of *Trpv4* renders these mice less sensitive to histaminergic pruritogens. We also topically applied TRPV4-specific small-molecule inhibitor, GSK205 (10, 33), to mouse epidermis. As a result, histaminergic, but not non-histaminergic, scratching was significantly attenuated (Fig. 1*E*), reiterating the conclusion derived from *Trpv4* cKO mice, namely that TRPV4 channels in epidermal keratinocytes are significant molecular actuators of organismal itch, driving the keratinocyte as itch generator cells. In addition, the experiments with topically applied TRPV4 blocking compound point toward a role for TRPV4 ion channel function as a critical contributor, not only reduced expression of the TRPV4 protein.

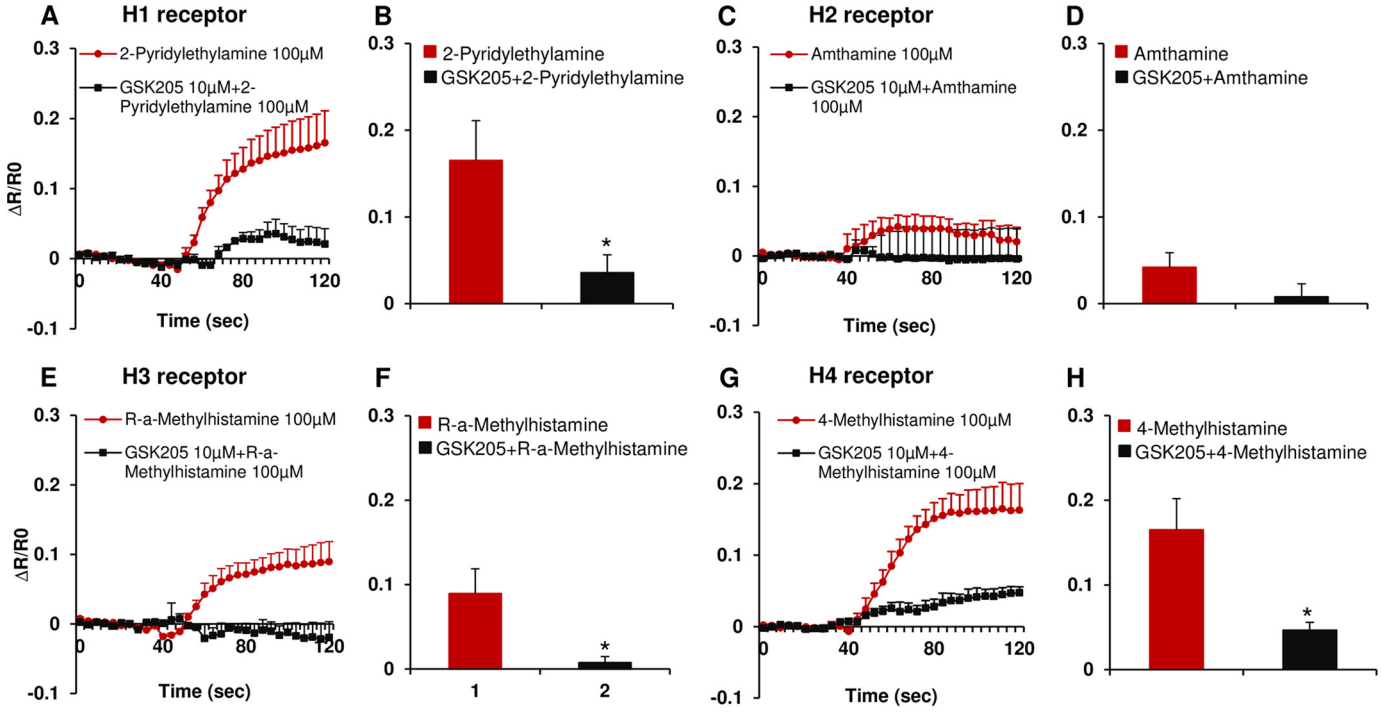

FIGURE 3. **Histamine receptor agonists induce Ca^{2+} influx in cultured murine keratinocytes via TRPV4 channels.** 2-Pyridylethylamine (selective H1 receptor agonist, *A* and *B*), and 4-methylhistamine (selective H4 receptor agonist, *G* and *H*), but not amthamine (selective H2 receptor agonist, *C* and *D*), evoked Ca^{2+} influx in murine keratinocytes, which was attenuated by pretreatment with TRPV4 inhibitor, GSK205. For selective activation of H3 receptor with *R*-α-methylhistamine (selective H3 receptor agonist, *E* and *F*), the increase were less robust. Two-tail *t* test was used for statistic analyses (*B*, *D*, *F*, and *H*; *, $p <$ 0.05 *versus* agonists). $n = 100–200$ cells/treatment.

TRPV4 Is Required for Histaminergic-dependent Pruritogens-induced Ca^{2+} Influx in Keratinocytes—These *in vivo* results raise the question of how the histaminergic pruritogens that we have used to evoke TRPV4-dependent scratching affect Ca^{2+} signaling in epidermal keratinocyes given that TRPV4 is known to function as a Ca^{2+}-permeable TRP channel in these cells (47). To address this specific question, we stimulated primary murine keratinocytes with the same pruritogens used *in vivo*, then asked whether inhibiting TRPV4 channel activity with a selective small-molecule inhibitor would attenuate any resulting Ca^{2+} transients. We first used the classic pruritogen, histamine, which resulted in a dose-dependent Ca^{2+} signal, strongly attenuated by two selective TRPV4 inhibitors, GSK205 and GSK219 (Fig. 2, *A–C*). We obtained a similar reduction of Ca^{2+} signal when stimulating cultured keratinocytes derived from *Trpv4*$^{-/-}$ pan-null mice, confirming the critical role of TRPV4 in Ca^{2+} influx downstream of histamine-receptor signaling. Given the significance of histamine for itch, we also elucidated the receptor subtype present in keratinocytes. Our results suggest histamine receptors of the H1, H3, and H4 subtype to be appreciably involved, not H2 receptors. This finding is in keeping with previously established expression patterns in keratinocytes (48–51) and illustrated in Fig. 3. These three histamine receptor subtypes signaled to TRPV4 as their selective stimulation led to an appreciable Ca^{2+} transient in primary keratinocytes that could be virtually eliminated by GSK205, as also illustrated in Fig. 3. Given the translational medical relevance of this finding, we recapitulated this experiment in primary human keratinocytes. Results are shown in Fig. 4, demonstrating a similar capability of histamine to evoke Ca^{2+} transients in primary human keratinocytes. These Ca^{2+} transients could be completely eliminated with GSK205, as shown in Fig. 4. Our findings indicate the TRPV4-mediated Ca^{2+} signal to rely on H1, H3, and H4 receptors. Congruency of mouse human histamine-TRPV4 signaling suggests an evolutionary conserved allergic inflammation mechanism that underlies integumental signaling from keratinocytes to sensory neurons.

Regarding Ca^{2+} signaling in response to other histaminergic pruritogens, we found that compound 48/80 (Fig. 2, *D–F*) and also the partial histaminergic ET-1 (Fig. 2, *G–I*) evoked Ca^{2+} transients in mouse primary keratinocytes in a dose-dependent manner that could be blocked with GSK205 and GSK219 or were dramatically reduced in keratinocytes derived from *Trpv4*$^{-/-}$ pan-null mice. Chloroquine, a non-histaminergic pruritogen, however, did not evoke a Ca^{2+} signal in keratinocytes (Fig. 2, *J–L*).

Taken together, we detected a TRPV4-dependent Ca^{2+} signal in cultured epidermal keratinocytes in response to histaminergic pruritogens. In view of our *in vivo* findings with *Trpv4* cKO mice, we reason that this Ca^{2+} signal is the cellular signaling correlate of histaminergic pruritogen-activation of TRPV4 channels in epidermal keratinocytes, which co-contributes significantly to scratching behavior *in vivo*.

GSK101, a TRPV4-selective Agonist, Elicits Scratching-behavior Dependent on TRPV4 Expression in Keratinocytes—Having recorded results that suggest (i) the *Trpv4* gene is necessary for scratching behavior in response to histaminergic pruritogens, (ii) TRPV4 channels in epidermal keratinocytes are necessary for these behaviors, and (iii) these channels in epidermal keratinocytes are activated by the G protein-coupled recep-

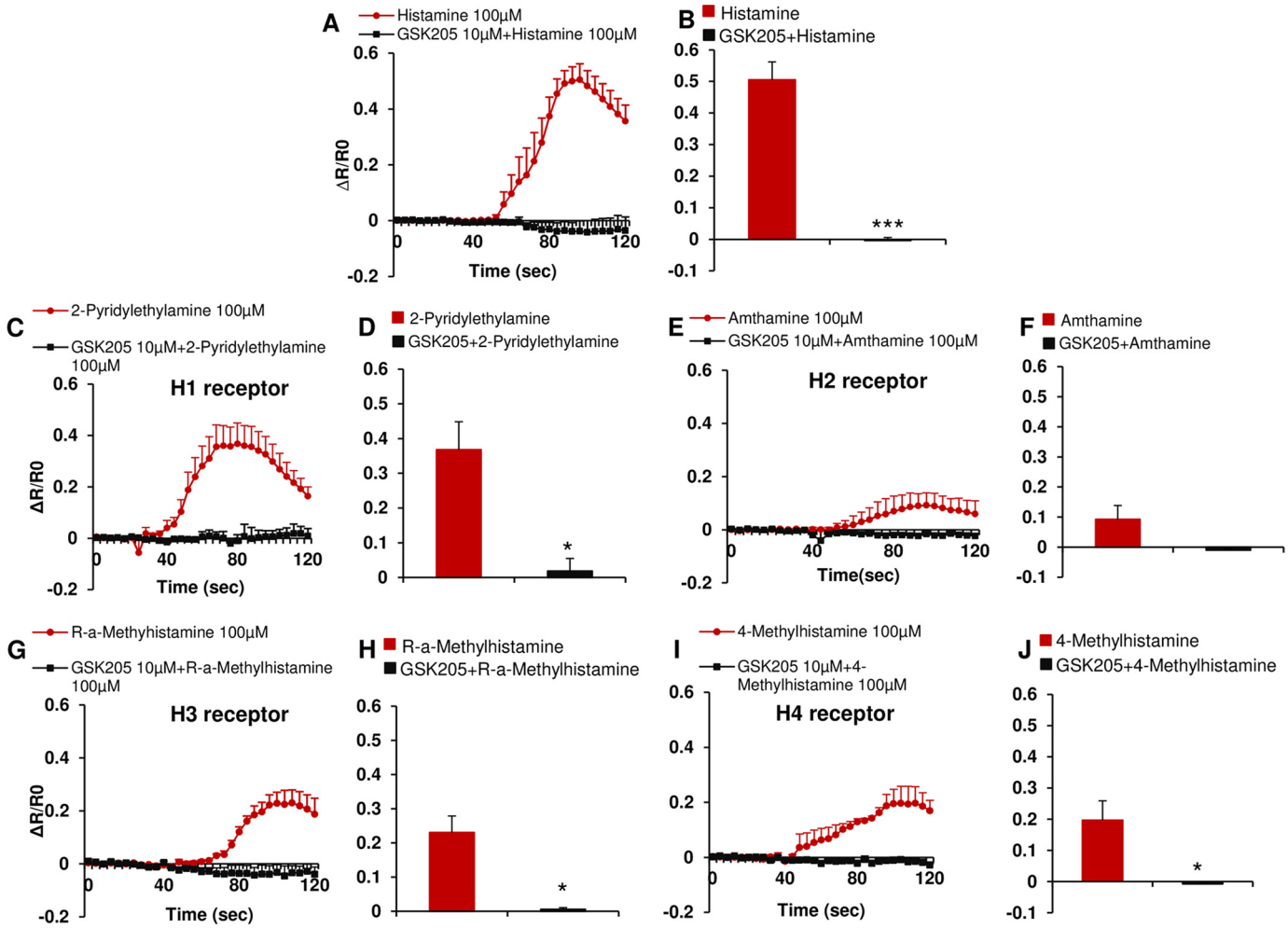

FIGURE 4. **Histamine receptor agonists induce Ca^{2+} influx in cultured human keratinocytes via TRPV4 channels.** Histamine (non-selective H receptor agonist, *A* and *B*), 2-pyridylethylamine (selective H1 receptor agonist, *C* and *D*), *R*-α-methylhistamine (selective H3 receptor agonist, *G* and *H*), and 4-methylhistamine (selective H4 receptor agonist, *I* and *J*), but not amthamine (selective H2 receptor agonist, *E* and *F*), evoked Ca^{2+} influx in a dose-dependent manner in human keratinocytes, which was attenuated by pretreatment with TRPV4 inhibitor, GSK205. A two-tail *t* test was used for statistic analyses (*A*, *D*, *F*, *H*, and *J*; ***, $p < 0.0001$; *, $p < 0.05$ *versus* agonists). $n = 100–200$ cells/treatment.

tors for the respective pruritogens, we next asked the question of sufficiency of TRPV4 activation for scratching behavior. We documented that increasing concentrations of small-molecule-selective TRPV4 activator, GSK101, evoked itch behavior with increasing frequency (Fig. 5*A*). In a *Trpv4* pan-null knock-out mouse, there was only a marginal, non-significant increase in scratching behavior in response to 65 ng of GSK101 *versus* WT or *Trpv4* pan-null knock-out control, indicating minimal off-target effects of GSK101 as a pruritogen *in vivo*. Thus, scratching behavior, at the organismal level, can be evoked by selective activation of TRPV4. Importantly, we verified that oil-induced *Trpv4* cKO mice scratched not differently from WT mice in response to GSK101. We also showed that *Trpv4* cKO mice induced with tamoxifen showed no significant increase in scratching behavior in response to intradermal injection of GSK101 *versus* WT or *Trpv4* cKO control (Fig. 5*A*). This finding is highly relevant because it suggests that direct activation of TRPV4 channels, expressed by keratinocytes, by intradermal injection of GSK101 leads to scratching behavior in live animals. This is critically dependent on TRPV4 expression by skin keratinocytes. Therefore, we decided next to assess keratinocyte response to selective activation of TRPV4. We did record a dose-response relationship of the resulting Ca^{2+} signal to GSK101 concentrations (Fig. 5*B*). This signal could be significantly attenuated when using two TRPV4-selective inhibitors, GSK205 or GSK219 (Fig. 5, *C* and *D*). Overall, these GSK101-related findings leave open the possibility of a co-contributory role of TRPV4 signaling in sensory neurons or other itch-relevant cells but together with our data, as presented in Figs. 1–4, make the case for an important role for TRPV4 in epidermal forefront signaling as a "pruriceptor-TRP" ion channel in epidermal keratinocytes.

ERK Signaling Downstream of TRPV4 in Skin Keratinocyte Is Essential for Histaminergic Itch—We next addressed the question of what signals intracellularly in epidermal keratinocytes, downstream of TRPV4-mediated Ca^{2+} influx. Choosing a candidate approach, we focused on mitogen-activated protein kinase signaling of the MEK-ERK pathway based on previous results in an epithelial cell type, upper airway respiratory epithelia, that also provides organismal barrier protection in which we demonstrated MEK-ERK activation in response to an environmental irritant (35, 52). We first probed whether there is

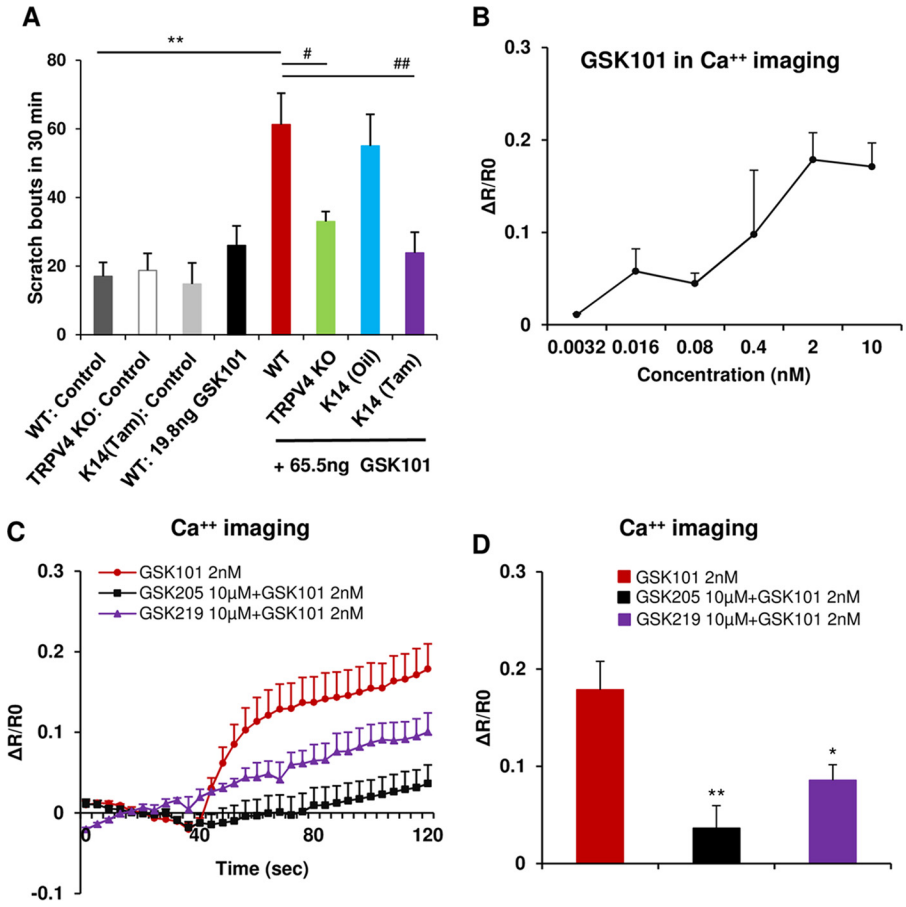

FIGURE 5. **GSK101 elicits scratching behaviors and triggers Ca^{2+} influx in cultured keratinocytes.** Animals injected with the TRPV4-selective activator, GSK101, displayed a significant scratching response that was attenuated in *Trpv4* pan-null mice. Importantly, scratching behavior depended on TRPV4 expression in keratinocytes, evidenced by a complete lack of response to GSK101 in *Trpv4* cKO mice (*A*, **, $p < 0.01$; #, $p < 0.05$; ##, $p < 0.01$). GSK101 evoked a Ca^{2+} response in a dose-dependent manner in keratinocytes (*B*). *C* and *D* illustrate the keratinocyte Ca^{2+} signal evoked by 2 nM GSK101 and its attenuation by TRPV4-selective inhibitors, GSK205 or GSK219 (*, $p < 0.05$; **, $p < 0.01$ *versus* GSK101). One-way analysis of variance with Tukey's post hoc test was used for *A*, and a two-tail *t* test was used for *D*. $n = 4–5$ mice/group (*A*) and $n = 150–300$ cells/treatment (*B–D*).

rapid ERK phosphorylation in response to histaminergic pruritogens. We recorded affirmative results at the 10-min time point in cultured keratinocytes and the 30-min time point in skin (dermis-epidermis) for all three histaminergic pruritogens tested, not for non-histaminergic pruritogen chloroquine (Fig. 6). The level of total ERK in both cultured cells and skin was not altered (data not shown). We then next addressed whether this rapid increase in ERK phosphorylation depends on TRPV4 by applying the TRPV4-inhibitor, GSK205. In primary keratinocytes, we observed a complete reversal to non-stimulated levels of pERK. In skin from mice challenged *in vivo*, we detected a similar response (Fig. 6). These findings suggest that pERK activation is down-stream of TRPV4-mediated Ca^{2+} influx. To determine the relevance of MEK-ERK phosphorylation for scratching behavior, we applied a selective inhibitor of MEK, U0126, in a topical formulation to skin. In response to histaminergic pruritogens we observed a significant anti-pruritic effect of topical U0126 when mice were intradermally treated and a lack thereof for non-histaminergic pruritogen, chloroquine (Fig. 7).

Discussion

In this study we describe a novel role for TRPV4 channels in histaminergic itch including ET-1-evoked itch. Importantly, TRPV4 expression and function in epidermal keratinocytes shows a robust contributory role to scratching behavior evoked by histaminergic pruritogens, not for the non-histaminergic chloroquine. This means that keratinocytes of the integument can function as itch generator cells and that TRPV4 plays a significant signaling role in these cells in mediating histaminergic itch. Importantly, direct activation of TRPV4 by intra-dermal injection of TRPV4 activator, GSK101, led to scratching behavior, which critically depended on TRPV4 expression in keratinocytes. This finding underscores the fundamental, hitherto unrecognized role of TRPV4 channels in epidermal keratinocytes in acute histaminergic itch. We recorded complementary findings in primary keratinocyte culture where we observed Ca^{2+} transients in response to the same diverse histaminergic pruritogens that elicit scratching behavior dependent on keratinocyte-TRPV4. We found this Ca^{2+} response to be mediated by TRPV4, which was activated by the respective pruritogens and their cognate keratinocyte-G protein-coupled receptors or directly via selective chemical activator. In epidermal keratinocytes, Ca^{2+} influx via TRPV4 elicits ERK phosphorylation as a downstream signaling event of the forefront pruritogen signaling discovered here. Topical transdermal inhibition of TRPV4 and its downstream kinase target, MEK,

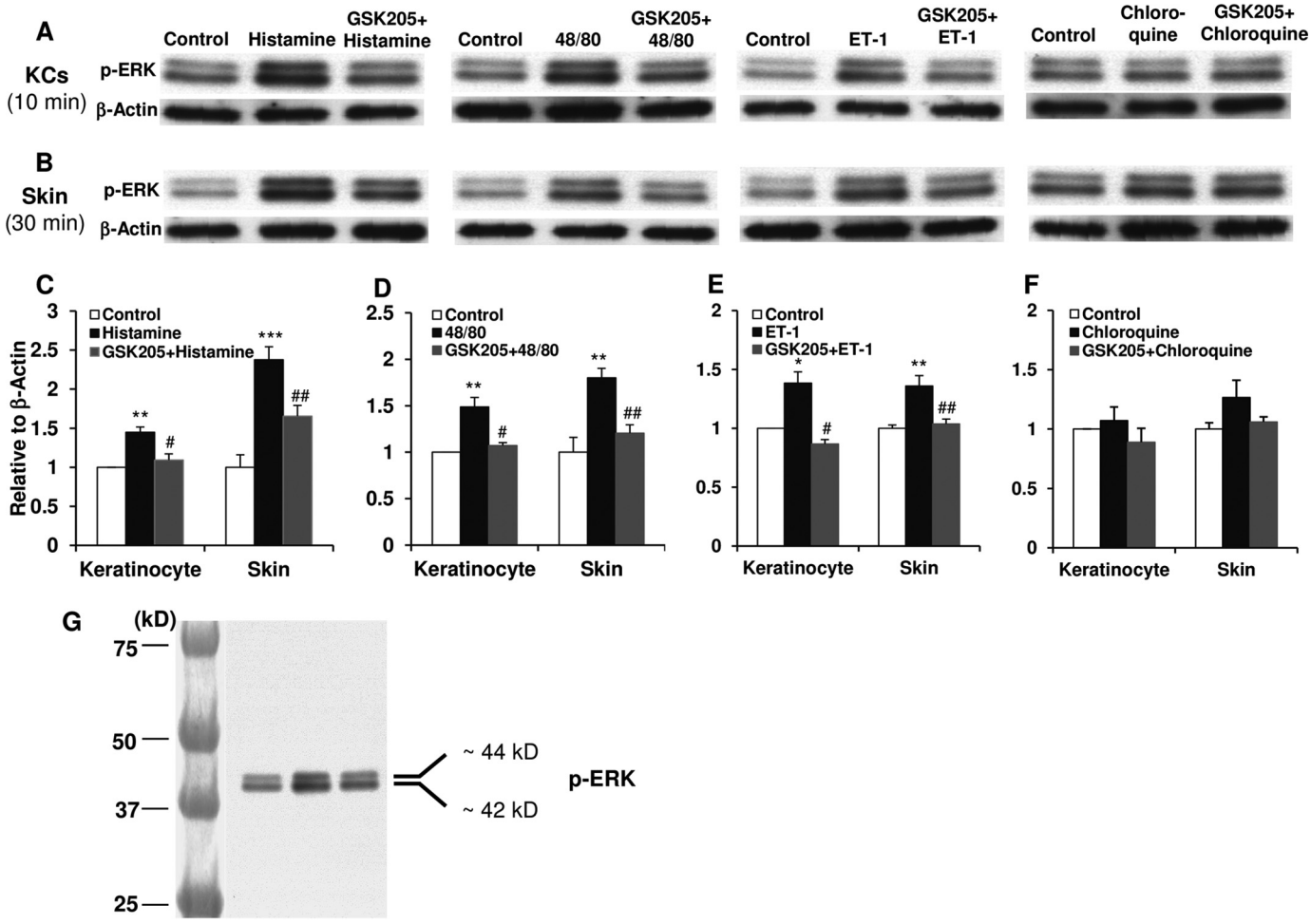

FIGURE 6. ***Trpv4* is essential for the increase of phospho-ERK (*pERK*) in skin keratinocytes (*KCs*) and the integument from live animals in response to histamine-dependent pruritogens.** Western blotting shows pERK expression in cultured keratinocytes (*A*) and nape skin (*B*). *Bar graphs* depict quantitation, demonstrating a significant increase of pERK evoked by histamine (*C*), compound 48/80 (*D*), and ET-1 (*E*), but not chloroquine (*F*) (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$: pruritogen *versus Control*). Importantly, a significant increase of pERK depends on TRPV4, demonstrated by its reduction to control levels by pretreatment with TRPV4-selective inhibitor GSK205 (*C–F*: #, $p < 0.05$; ##, $p < 0.01$: GSK205+pruritogen *versus* pruritogen). *G* shows Western blots of pERK with a standard molecular mass marker. Two-tail *t* test was used for statistic analyses. $n = 3$ cultures/group for the cultured keratinocytes and $n = 5\sim6$ mice/group for skin.

which functions upstream of ERK phosphorylation, both showed robust anti-pruritic efficiency in mice challenged with histaminergic pruritogens. Activation of MEK-ERK signaling in keratinocytes is also known from non-itching skin conditions (53, 54). We speculate that MEK-ERK activation by TRPV4 could be the important explanatory difference. This hypothetic concept will have to be tested in future studies. Our results argue for a novel translational medical path of topical treatments to skin that target molecularly defined signaling mechanisms that modulate sensory transduction (55).

In this paper we focus first on acute itch and the role of histaminergic pruritogens. Additional pruritogens need to be studied in the future, with particular focus on chronic itch, a medically relevant condition because of its prevalence and substantial unmet medical need. A central question that we have not addressed in this study is, What specific cellular and molecular mechanisms of cell-to-cell communication do epidermal keratinocytes employ? How does the histaminergic pruritogen-G protein-coupled receptor-TRPV4-Ca^{2+}-pERK pathway evoke these signaling mechanisms, and how does this trigger pruriceptor sensory neurons to transmit the signal toward the nervous system? We hypothesize that soluble factors play such roles, possibly proteins, peptides, small-molecule phospholipids, and lipid molecules that are released from keratinocytes to affect innervating peripheral nerve endings of pruriceptor neurons perhaps either via direct keratinocyte nerve fiber signaling or rather indirectly via involvement of immune, vascular, and other adjacent cells.

A recent study examined the response of spinal cord dorsal horn relay neurons to intradermal injection of ET-1 (16). The study reports that ET-1-sensitive neurons respond to multiple modalities yet that >50% respond to spinal superperfusion of the peptide bombesin, which can activate spinal gastrin-releasing peptide receptors known to function in itch circuits, thus identifying these neurons as part of a specific itch circuit that relies on gastrin-releasing peptide for transmission. When viewing these results together with our current findings, an interesting formation of an ET-1-responsive itch circuit emerges that has its origin with ET-1-responsive keratinocytes that use TRPV4 as critical Ca^{2+} influx mechanism in response

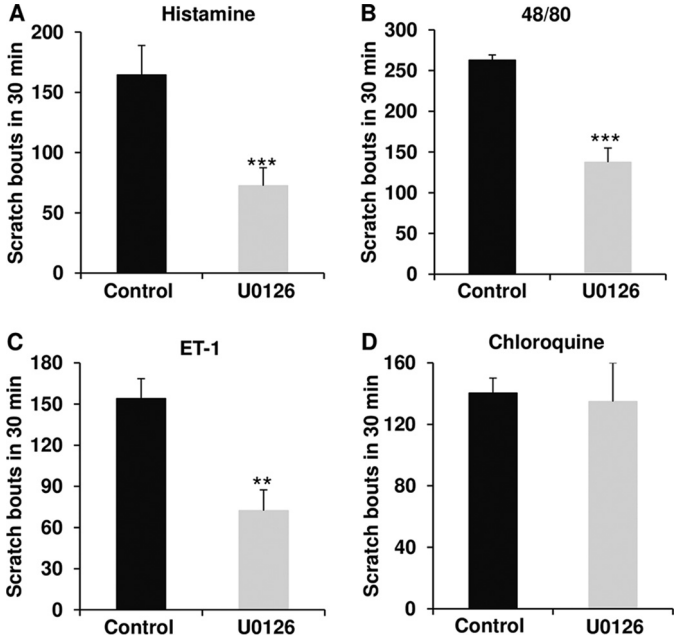

FIGURE 7. **ERK signaling downstream of TRPV4 is relevant for histaminergic pruritogen-evoked scratching behaviors.** Histamine (*A*), compound 48/80 (*B*), ET-1 (*C*), but not chloroquine (*D*) cause scratching behaviors that are significantly attenuated in mice topically pretreated with the MEK selective inhibitor U0126 (**, $p < 0.01$; ***, $p < 0.001$ *versus Control*). Two-tail *t* test was used for statistic analyses. $n = 5–6$ mice/group.

to ET-1 receptor-A activation (10). Subsequently these ET-1-responsive keratinocytes activate innervating peripheral sensory neurons, which need to be more precisely defined in future studies, which in turn relay to spinal cord dorsal horn neurons, more than half specifically dedicated to itch-relay via neurotransmission that relies on gastrin-releasing peptide (16).

In another recent study from these same authors, *Trpv4* pan-null mice were reported to scratch less in response to intradermal injection with serotonin (56). The authors report that they did not see different scratch behavior in response to histamine. In this respect, their results differ from our results in *Trpv4* pan-null mice. This discrepancy may be related to technical detail such as difference in doses of pruritogen, animal ages, and behavioral assessment methods. Of note, the originating line of mice used is identical between our current and the referenced study. We believe that this seemingly perplexing discrepancy can possibly be resolved in future studies that focus on the influence of genetic background on nocifensive and pruritic behavior and, more importantly, on the impact that epigenetic regulation might play. Of note, different phenotypes of identical lines of *Trpv4* pan-null mice, propagated in different laboratories, have been reported previously (57, 58). Importantly, we want to stress that the focus of our present investigation is the distinct contribution of TRPV4 channels in keratinocytes to histaminergic itch, a subject of basic science and translational-medical relevance that is not directly approached in Akiyama *et al.* (56).

With TRPV4 expression in the primary sensory neuron and in the CNS in neurons and glial cells established, which roles do neural and neuronal TRPV4 play in itch transduction, transmission, and plasticity? Whereas these questions remain to be answered in future studies, we wish to reiterate the key concept of TRPV4 as forefront pruriceptor TRP channel functioning in epidermal keratinocytes, to drive the organismal scratch response. This concept bears the translational-medical mandate, as mentioned, to develop selective anti-TRPV4 treatments that can be applied topically and that will also have to be inert regarding epidermal cell growth regulation in view of recent findings of attenuated TRPV4 expression in skin epithelial malignancies (59).

Pruriceptor-TRPs comprise TRPA1, TRPV1, TRPV3 (40, 60–68), and now also TRPV4. Their possible mechanisms of interaction and the respective cellular locale will be attractive subjects for the following chapters in this intriguing story. Possibly, human genetic variation in the respective *TRP* pruriceptor genes might be relevant for different itch susceptibilities both for physiologic and pathologic forms of itch.

Author Contributions—Y. C. and W. B. L. conceived and coordinated the study and analyzed the data. Y. C., Q. F., and Z. W. designed, performed, and analyzed the experiments. J. Y. Z. contributed reagents. Y. C., J. Y. Z., A. S. M., R. P. H., and W. B. L. drafted and revised the article. All authors reviewed the results and approved the final version of the manuscript.

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Hot Topic Review

Pleiotropic function of TRPV4 ion channels in the central nervous system

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New Findings

- **What is the topic of this review?**
  In this concise review, we highlight insights into the role of transient receptor potential, vanilloid type 4 (TRPV4) ion channels in the CNS, results that have been contributed over the last 16 years since the initial discovery of the channel.
- **What advances does it highlight?**
  TRPV4 has been found to function in neurons, astroglia and microglia, both in physiological (e.g. astrocytic neurovascular coupling, neuronal membrane potential at physiological temperature) and in pathological conditions (e.g. mechanical trauma), so far recorded as exciting findings in need of more in-depth mechanistic clarification.

Transient receptor potential, vanilloid type 4 (TRPV4) ion channels are osmo-mechano-TRP channels, with pleiotropic function and expression in many different types of tissues and cells. They have also been found to be involved in pain and inflammation. Studies have focused on the role of TRPV4 in peripheral sensory neurons, but its expression and function in central nervous glial cells and neurons has also been documented. In this overview, based on the senior author's (WL) lecture at the recent recent joint meeting of APS/The Physiological Society in Dublin, we concisely review evidence of TRPV4 expression and function in the CNS and how TRPV4 function can be modulated for therapeutic benefit of neuropsychiatric disorders. Novel TRPV4-inhibitory compounds developed recently in the authors' laboratory are also discussed.

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Introduction

Transient receptor potential, vanilloid type 4 (TRPV4) ion channels (Liedtke *et al.* 2000) are also expressed in cells of the CNS, including neurons, astrocytes and microglia (White *et al.* 2016). Cell physiology experiments indicate that TRPV4 channels can function as Ca^{2+}-permeable cation channels that are gated by various stimuli, such as cell swelling, low pH, mechanical stress and temperature (Liedtke & Kim, 2005; Liedtke, 2008; Guilak *et al.* 2010; McNulty *et al.* 2015; White *et al.* 2016). Whether this currently established functional profile is particularly relevant for the role that TRPV4 plays in the CNS remains to be determined. As of today, 16 years after its initial description (Liedtke *et al.* 2000; Strotmann *et al.* 2000), studies on specific functions of TRPV4 in various regions of the CNS are not further advanced than the early stages of exploration. Slow progress could be attributable to low levels of TRPV4 expression in certain cell types within the CNS, yet still contributing to important function. TRPV4 could function both developmentally and postdevelopmentally as a sensor-signalling molecule. Despite this lack of a clear picture, TRPV4 appears to function in glial cells and neurons in basic physiological

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Experimental Physiology

and in specific pathological conditions, which are reviewed concisely here. One particular focus of this article is to relate known findings on TRPV4 in the CNS to what this might mean for medicinal translational purposes.

Role of TRPV4 in astrocytes

TRPV4 channels have been found to be expressed in astrocytes. These channels were localized in the plasma membrane of astrocytes and exhibited activation in response to a selective agonist, showing a typical outwardly rectifying cation current (Benfenati *et al.* 2007). As a Ca^{2+}-permeable channel, TRPV4 was shown to influence neurovascular coupling through Ca^{2+}-induced Ca^{2+} release from inositol trisphosphate receptors in astrocytic endfeet (Fig. 1*A*; Dunn *et al.* 2013). Studies indicate that astroglial cells can modulate neuronal excitability in the hippocampus, cortex and hypothalamus and that TRPV4 might function as a key player in this excitation (Simard & Nedergaard, 2004; Shibasaki *et al.* 2014). In the hippocampus, for example, TRPV4 is highly expressed in astrocytes of the CA1 region, and its enhanced expression in this region coincides with the development of astrogliosis (Shirakawa *et al.* 2010; Butenko *et al.* 2012).

Other studies suggest that TRPV4 may form a complex with aquaporin-4 (AQP4/TRPV4) and function in control of cell volume in astrocytes and be involved in regulating interstitial tonicity in the brain, as well as in formation of brain oedema (Benfenati *et al.* 2011). Furthermore, TRPV4 channels in hippocampal astrocytes were considered to function in oxidative stress-induced cell damage (Bai & Lipski, 2010). Other evidence suggests that astrocytic TRPV4 may be involved in neuronal toxicity evoked by the Alzheimer's disease-associated peptide, $A\beta_{40}$ (Fig. 1*C*; Bai & Lipski, 2014). Astrocytes may therefore play crucial roles in the homeostatic regulation of the CNS not only in physiological but also in pathological conditions. TRPV4 channels expressed in astroglial cells were also shown to mediate infrasound-induced neuronal injury (Fig. 1*B*), a model for blast-induced traumatic brain injury, impairing learning and memory in rats (Shi *et al.* 2013). This last study also indicated that injury was more pronounced in the hippocampal CA1

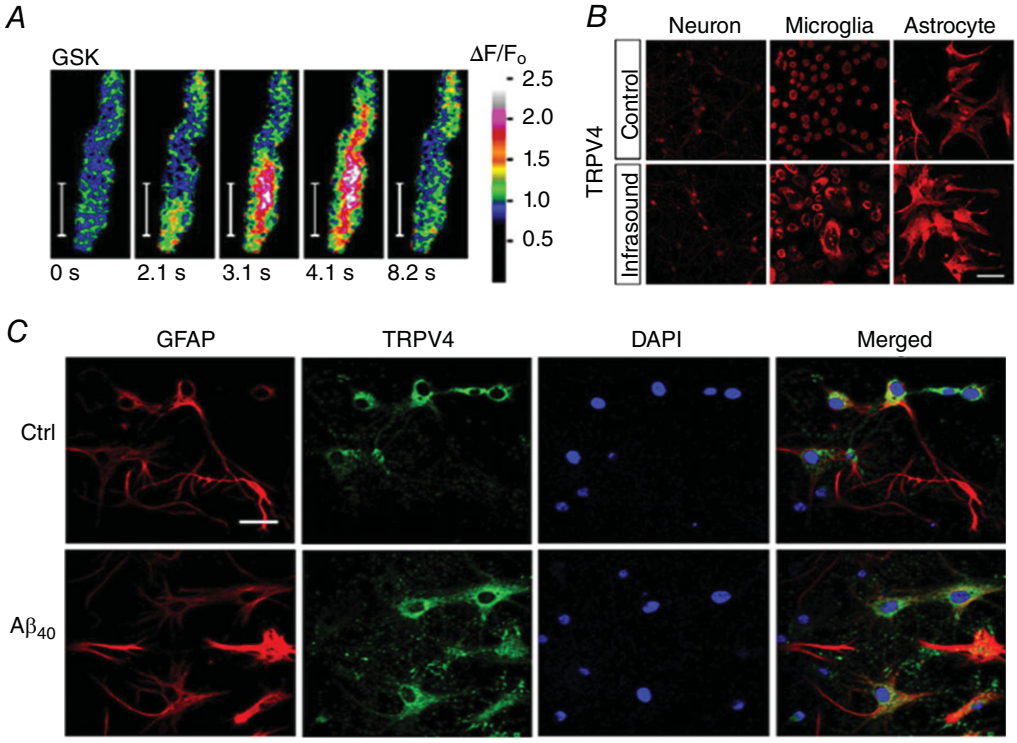

Figure 1. TRPV4 in astrocytes in physiological conditions and upregulated in response to injury
A, time course of an astrocytic endfoot Ca^{2+} transient evoked by selective stimulation of TRPV4, using selective activator compound GSK101; from Dunn *et al.* (2013). *B*, infrasound-induced mechanical stress response of cultured CNS lineages; note upregulation of TRPV4 in response to infrasound in microglia and astrocytes; from Shi *et al.* (2013). *C*, exposure of cultured astrocytes to Alzheimer's disease-associated amyloid peptide $A\beta_{40}$ induces increased expression of astrocytic intermediate filament protein, GFAP, and TRPV4 ion channels; from Bai & Lipski (2014).

region, where astrocytic and microglial activation was observed prior to neuronal apoptosis. Inhibition of TRPV4 protected neurons from infrasound injury by decreasing the expression levels of glial cell-released pro-inflammatory cytokines interleukin-1β and tumour necrosis factor-α.

Role of TRPV4 in microglia

Interestingly, microglial activation after an injection of lipopolysaccharide into the mouse cerebral ventricle was inhibited by concurrent administration of a TRPV4 agonist, 4α-phorbol 12,13-didecanoate (Shirakawa *et al.* 2010; Konno *et al.* 2012). Shirakawa *et al.* (2010), Konno *et al.* (2012) and Shi *et al.* (2013) describe seemingly opposing roles of TRPV4 in microglial activation in response to mechanical stimulation *versus* chemical stimulation via lipopolysaccharide, indicating the important but complex role of TRPV4 in microglia, which is currently underexplored, thus in need of future study.

Role of TRPV4 in CNS neurons

In hippocampal neurons, the influx of cations through TRPV4 channels at physiological temperature may control neuronal excitability by regulation of the resting membrane potential (Shibasaki *et al.* 2007; Shibasaki *et al.* 2015*a*; see also Shibasaki *et al.* 2015*b*), and possibly also in other neurons, with wide implications for brain functioning, manifested in several abnormal behavioural parameters in *Trpv4*$^{-/-}$ mice, as recently documented (Shibasaki *et al.* 2015*a*). It is possible that TRPV4 expression and function in neurons might play a significant role in seizures. In larval zebrafish, for example, febrile seizure-related neural activity, which was triggered by an increase in brain temperature, was shown to be blocked by a TRPV4 antagonist and not by GABA re-uptake inhibitors (Hunt *et al.* 2012). Furthermore, increased TRPV4 expression in cortical lesions of patients with focal cortical dysplasia, a known form of therapy-refractory epilepsy, indicates that TRPV4 might contribute to cortical malformation and maldevelopment, which may facilitate epileptogenesis (Chen *et al.* 2016). As an important qualifier, indicative of the superficial level of inquiry in this field, changed expression levels do not conclusively substantiate any association with disease pathogenesis. In other studies, mutations in *TRPV4*, hereditary TRPV4 channelopathies, were shown to cause excessive Ca^{2+} influx related to motor nerve axonopathy and spinal muscular atrophy (Fecto *et al.* 2011; Jang *et al.* 2012). Although the human genetics of these disorders are clear, it is unclear why some *TRPV4* channelopathy mutations cause skeletal malformations, whereas other mutations detrimentally affect spinal motoneurons. For the former, mechanistic evidence regarding a feasible pathomechanism has been provided, at least an initial inroad (Leddy *et al.* 2014*a*,*b*). In contrast, for the sequence of events between excess Ca^{2+} influx and subsequent motoneuron dysfunction, this has hitherto remained elusive.

Interestingly, in ageing, age-related expression changes of TRPV4 have been reported in pyramidal cortical neurons, thalamus, basal nuclei of the cerebellum and in the spinal cord (Lee & Choe, 2014). The expression of TRPV4 in neurons in these brain regions could be the basis for (mal)function of TRPV4 in pathological conditions. For example, findings by Lee *et al.* (2012) on age-dependent expression of TRPV4 channels may be indicative of the role of TRPV4 in the pathogenesis of age-related neurodegenerative diseases. Neuronal TRPV4 channels may therefore be an important therapeutic target for cognitive, motor and ageing-related disorders. TRPV4 inhibitors have been described and were recently described as orally available compounds (Jia *et al.* 2004; Krause *et al.* 2005; Phan *et al.* 2009; Vincent *et al.* 2009; Morty & Kuebler, 2014; Feetham *et al.* 2015; Qi *et al.* 2015) and also as 'dual inhibitors' of TRPV4 and TRPA1 (Kanju *et al.* 2016), which might be an attractive possibility given the postulated function of astrocytic TRPA1 (Lee *et al.* 2012; Shigetomi *et al.* 2012, 2013; Wei *et al.* 2016). Such inhibitors, if they are intended for oral use, will have to pass through the blood–brain barrier (although, for example, the blood–brain barrier is more penetrable in certain CNS disorders, such as multiple sclerosis). As an alternative, if TRPV4 or TRPV4/TRPA1 (dual) blockers can potently treat more severe neuropsychiatric illnesses safely and effectively, intrathecal administration remains a plausible alternative (e.g. for therapy-refractory high-intensity chronic pain, note its treatment with intrathecal ziconotide; McDowell & Pope, 2016).

Although an interesting topic, the role of TRPV4 in the CNS in systemic osmoregulation and hydromineral regulation is not reviewed here (but see Liedtke & Friedman, 2003; Janas *et al.* 2016), mainly because it was covered in a separate presentation at the recent joint meeting of APS/The Physiological Society in Dublin, Ireland, and therefore not covered in the overview talk by the senior author (WL). This qualifier also applies to the important topic of the role of TRPV4 in the CNS in the choroid plexus (Takayama *et al.* 2014), first described by Liedtke *et al.* (2000).

Conclusion

In this concise review, we highlight the important, yet currently underexplored role of TRPV4 in the CNS in glial cells and neurons, which has so far not been met with appropriate attention and matching investigative scrutiny.

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Title: **Regulation of pain and itch by TRP channels**

Subtitle: TRP = targeted relief of pain or targeted relief of pruritus? Add “pain-TRP” and “itch-TRP” to the lexicon!

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Contents

1. Introduction

Pain is a vital, survival-enhancing protective mechanism from harm that can potentially threaten animals' organismal integrity, their survival and capability to generate offspring. Pain relies on a nervous system with a dedicated sensory subdivision. Pain with a unique conscious sensation that can be described, by definition, is exclusively human. However, vertebrates also have sophisticated pain mechanisms, including pain-related behavior and activation of neural pathways dedicated to sensing harm that leads to acute withdrawal or other protective behaviors. Pain-like behavior and the respective neural circuits are also present in invertebrates with a nervous system that has sufficient organization, e.g. *Drosophila* and *C. elegans*. In humans, there are numerous pathological conditions, such as diabetes, viral infections, metabolic, toxic and traumatic nerve

damage, and inflammation of neural structures, which can produce non-relenting pain which we can view as one form of pathologic pain. Chronic pain has no apparent useful purpose for organismal survival, and represents a challenge for effective remedial measures. Of note, pathologic pain can also manifest as purely episodic, e.g. migraine or cluster headaches, trigeminal neuralgia, peripheral or visceral neuralgic pain, whereby inter-episode pain is minimal or absent, interrupted by subjectively disruptive to destructive episodes/ attacks of completely inappropriate pain.

Following a noxious stimulus (from externally or internally), primary sensory neurons in dorsal root or trigeminal ganglion dedicated to detecting damage – nociceptor neurons - are activated by transduction of the noxious stimulus, which then leads to action potential propagation along the axons of the primary afferent fibers (C and Aδ fibers) to the nerve terminals found in laminae I and II of the dorsal horn in the spinal cord, or the respective spinal nucleus of the trigeminal system. These nerve terminals release neurotransmitters such as glutamate, substance P, and calcitonin gene related peptide (CGRP) to activate postsynaptic receptors located in spinothalamic tract neurons [1]. The neural afferents that project to the thalamus constitute a neural circuit that results in pain perception via projections to the cortex of the brain. A variety of receptors, iono-tropic, G-protein coupled and receptor-tyrosine kinase related, transduce, propagate and modulate pain signals, setting up an array of potential targets for rational modulation of pain for remedial purposes.

Nociceptive primary neurons send signals from the periphery or from internally (e.g. visceral pain, pain of myocardial infarction, airway, musculo-skeletal origin), via the afferent fibers, to specific cortical regions in a somato-topic manner. Nociceptive relay

neurons connect the spinal cord (for DRG-mediated pain) and brain stem (for trigeminal pain) to the brain, thus serving as mediators in painful stimulus transmission between the central and peripheral nervous systems (CNS and PNS). These neurons sustain their function via nerve impulse transmission machinery that is made up of a number of different receptors including ion channels distributed along the neurons' projections and in their somata. In particular in the periphery, these receptors constitute the molecules that detect noxious stimuli, transforming them into electrical signals for routing to the CNS [2]. Of medical importance, allodynia is defined as a non-painful stimulus evoking pain, e.g. a subtle draft of air on the skin, luke-warm water, or mechanical pull of the earlobe by an earring or the skin of the scalp by a pony-tail, indicative of neural pain circuits driven by non-nociceptive primary afferents.

A relevant gene family that is involved in pain sensory function is the transient receptor potential (TRP) channel super-family. This family is made up of ion channel proteins that function as non-selective cation-permeable channels that can conduct calcium, for virtually all members (28 in mammals) [3]. In general, TRP channels have fascinated researchers, clinicians, drug developers and generally-interested scholars because they can function as molecular sensors of multiple physical and chemical stimuli, including changes in pH, chemical irritants including pungent peppers, wasabi, mustard, and menthol, also thermal, mechanical, osmotic and actinic (radiation) cues. The TRP super-family is composed of 28 members divided into six subfamilies, classified as canonical (TRPC), vanilloid (TRPV), ankyrin (TRPA), melastatin (TRPM), polycystin (TRPP), and mucolipin (TRPML) [4]. Reflecting on the acronym, it has also been proposed whether TRP

could not refer to “targeted relief of pain”. We propose here the “P” to be ambiguous and stand for “pain/pruritus”.

The TRP channel structure, now elucidated by several high-res cryo-EM structures, consists of four subunits, each containing six transmembrane segments (S1-S6). A domain between S5-S6, containing a hydrophilic loop, forms the ion-conducting pore and the selectivity filter [5, 6]. The most highly variable regions within TRP channels are the carboxyl and amino terminal ends. The ankyrin repeat domain is located within the amino terminus of TRP channels. However, the TRP box, which is a conserved six amino acid sequence found in the TRPC, TRPM, TRPA, and TRPV subfamilies, is located at the carboxyl terminus. Several studies have shown that this region can be important for ligand binding. In addition to the ankyrin repeat and TRP box domains, TRP family members contain other domains, including the calcium binding EF-hand domain, PDZ domains that anchor the receptor proteins in the membrane to cytoskeletal components, or NUDIX (hydrolase of a **nu**cleoside **di**phosphate linked to some other moiety, ***X***) domain. These domains are found in various TRP family members [6]. Diversity in their domain structure indicates that TRP channels can possibly form complexes with multiple proteins involved in different cellular processes, facilitating their participation in multiple cellular signaling processes.

20 years after the first description of TRPV1 as the first family member with postulated and later verified link to pain (followed by TRPV4, 3 years thereafter), the evidence has become more and more robust that TRP channels function in physiologic and pathologic pain. In this chapter, we elaborate on some relevant highlights related to the TRPA, TRPM, and TRPV subfamilies and their members’ roles in both physiologic and pathologic

pain and also itch. In addition, we propose to refer to TRP ion channels involved in pain transduction and transmission as a functional subfamily of “pain-TRPs”, following the previously claimed functional subfamilies of “thermo-TRP”, and also “mechano-TRP”, previously coined by one of us [7], and like-wise refer to TRP ion channels involved in itch as “itch-TRPs”.

2. TRPA subfamily

The TRP ankyrin (TRPA) subfamily is named for the large number if ankyrin repeats contained in the cytosolic N-termini of its members. The mammalian genome contains a single TRPA gene, TRPA1 [8]. The TRPA1 protein has 14 ankyrin repeats and forms a nonselective cation channel permeable to calcium [9, 10]. TRPA1 channels are highly expressed in peripheral nociceptors, with some studies reporting additional sites of expression. TRPA1 is functionally involved in [11-13]a variety of physiological or cellular processes in humans including nociception, especially in response to chemical irritants, itch, neurogenic inflammation and, potentially, thermosensation [12, 14-18], and may possibly affect immune system and vascular function [19].

2.1. TRPA1 is a universal chemo-irritant receptor and involved in neuropathic cold-pain

TRPA1 is a polymodal nocisensor gated by a wide range of chemical irritants. It is present in a subpopulation of Aδ- and C-fiber nociceptive sensory neurons in dorsal root, trigeminal and vagal (nodose, jugular) ganglia. TRPA1 is activated by pain-inducing natural products, including mustard oil (allyl isothiocyanate, AITC), the pungent ingredient

in mustard, wasabi and horseradish, cinnamaldehyde from cinnamon and pungent compounds in garlic (allicin) and onions (diallyl disulfide) [16, 20-22]. These natural products evoke pain-related behaviors in animals, and pain and irritation in humans (Figure 1). The chemical reactivity and structural diversity of these compounds suggested that TRPA1 does not fit into the mold of the traditional definition of a pharmacological receptor, but may in fact be a chemical reactivity detector, acting as a chemical warning sensor that translates chemical reactivity into a pain signal. Indeed, reactive cysteine residues in the intracellular N-terminal domain of TRPA1 were found to be essential for the majority of electrophilic agonists to gate the ion channel [23, 24]. More recent studies discovered additional sites within the ion channel protein, including lysine residues, that also contribute to reactivity detection [24-26]. TRPA1 is also sensitive to non-reactive irritating natural products, including carvacrol (from clove), thymol (from thyme), gingerol (from ginger) and menthol (mint) [27-29].

Chemosensory nerve endings in the cornea, the nose and the larynx constantly monitor the environment for airborne chemical threats, initiating defensive reflexes such as lachrymation, sneezing and cough. The upper and lower respiratory tract are innervated by primary afferent C-fibers and Aδ-fibers. Airway-innervating trigeminal and vagal ganglia all show appreciable TRPA1 expression [20, 30-32]. A significant number of airborne chemicals including industrial pollutants, but also natural substances, induce airway irritation by activating pulmonary/airway innervating sensory neurons in a TRPA1-dependent manner [28, 33-36]. TRPA1 expressed in airway-innervating neurons mediates acute irritation responses to tobacco smoke and smoke from fires, activated by the smoke irritants, acrolein and croton aldehyde [28, 32, 36-38].

The most potent TRPA1 agonists identified so far are tear gas agents such as 2-chloroacetophenone (in CN tear gas), 2-chlorobenzalmalononitrile (in CS tear gas) that activate TRPA1 in the low nanomolar or picomolar range [39, 40]. TRPA1-deficient mice lack acute nocifensive responses to these extremely painful agents [40]. TRPA1 is also sensitive to oxidizing chemical exposures, including chlorine gas, hydrogen peroxide and ozone [32, 34]. Respiratory reflex responses to these exposures are absent in TRPA1-deficient mice [32, 34] . TRPA1 activity is also contributing to the toxicological effects of particulates and metals such as zinc, cadmium and copper, underscoring TRPA1's essential role in the detection of toxic environmental agents [41-43]. Airborne environmental exposures such as ozone, particulates and acrolein affect cardiovascular function even at very low levels, initiating changes in blood pressure and heart rate. TRPA1 activation by inhaled diesel exhaust and acrolein was demonstrated to initiate cardiac arrhythmia in mice and rats, likely through changes in vagal sensory-autonomic control of cardiac function [44, 45].

While the function of TRPA1 as a chemosensor is firmly established its role in thermal sensing remains a matter of debate. In mammals exposures to cold temperatures below ~15°C can elicit pain. TRPA1 was initially identified as a sensor for noxious cold temperature, however, this study under-reported the prevalence of TRPA1 expressing sensory neurons that turned out to be much larger than the population responding to noxious cold temperatures [20, 46]. Studies in TRPA1-deficient mice reported contradictory data, some observing no difference in noxious cold induced nocifensive behavior [36, 47-49], also when TRPA1-expression was specifically ablated in sensory neurons or inhibited by a selective antagonist [50, 51]. The complete ablation of TRPA1-expressing sensory nerves

did not change the sensitivity of trigeminal nerves to cold [52]. Other studies detected diminished responses to cold stimuli in TRPA1-deficient mice, however, mice were exposed to freezing temperatures that may damage tissue and induce inflammation [12, 53]. Thermal sensitivity of TRPA1 is clearly species-specific, with some reports finding that human and other primate orthologs lack cold sensitivity, while others observed bi-modal (cold- and heat-) sensitivities [11, 54-56].

TRPA1 channels are highly sensitive to Ca2+ that has bimodal effects on channel function. Ca2+ potentiates and activateses TRPA1, but is also essential for TRPA1 desensitization. , and can be indirectly regulated by G protein-coupled receptors (GPCRs), e.g. bradykinin receptors and proteinase-activated receptors (PAR-2) or CGRP-signaling [57].

Less controversial is the role of TRPA1 in cold allodynia associated with inflammatory and neuropathic conditions, including chemotherapy-induced neuropathies subsequent to treatment with platinum or taxol therapeutics [25, 58-61] In this case, TRPA1 may serve as an amplifier increasing the excitability of cold-sensitve nerves [58, 62]. A proposed mechanism involves reactive oxygen species (ROS) signaling induced by cooling [63]. Inhibition of hydroxylation of a proline residue in a human TRPA1 N-terminal ankyrin repeat either by mutation or using a prolyl hydroxylase inhibitor can potentiate the cold sensitivity of TRPA1 in the presence of hydrogen peroxide. The same study showed that inhibiting prolyl-hydroxylase in mice triggers TRPA1 sensitization which is sufficient to sense cold-evoked ROS, hence causing cold hypersensitivity. Furthermore, this mechanism is thought to underly the acute cold hypersensitivity induced by the chemotherapeutic agent oxaliplatin or its metabolite oxalate [63].

TRPA1 has also been implicated in diabetic neuropathic pain, caused by chronic reactive chemical stress due to metabolic imbalance. In rodent models TRPA1 inhibitors were shown to protect nerves from peripheral degeneration and reduced nocifensive responses [64, 65]. Methyl glyoxal, a reactive metabolic product increased in diabetics, is a TRPA1 agonist that may contribute to chronic heightened channel activity, leading to increased pain sensation, Ca2+ overload and subsequent peripheral degeneration [66, 67].

2.2. TRPA1 in inflammatory pain

TRPA1 sensitization leads to hyperalgesia in response to various stimuli. TRPA1's pathophysiological role in inflammation is based on its ability to activate in response to various mediators and metabolites produced under inflammatory conditions. Pro-inflammatory mediators released from non-excitable cells include: ATP, bradykinin (BK), prostaglandins (PGs), leukotrienes, histamine, tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), proteases, and glutamate.

BK indirectly activates TRPA1 channel to mediate the pain and inflammatory response. This requires BK to interact with its receptor (B2R) which further activates the PLC-dependent signaling pathway, and promotes PIP_2 hydrolysis to produce inositol triphosphate (IP_3) and diacylglycerol (DAG). Both lipids IP_3 and DAG can activate TRPA1 [16]. Trpa1-/- mice have reduced thermal and mechanical pain responses to intraplantar injection of BK or AITC [36].

Agents such as Complete Freund's Adjuvant (CFA) are widely used to evoke inflammatory pain equivalents in preclinical models. This leads to increased expression of TRPA1 in

DRG neurons [30]. Furthermore, TRPA1 antagonist HC-030031 reduces nocifensive behaviors induced by paw injection of formalin and suppress mechanical hyperalgesia in the CFA model of inflammatory pain [68].

Human TRPA1 is also activated by acidosis which occurs in tissue ischemia such as myocardial infarction or peripheral vascular occlusive disease [69]. However, initial attempts to combat acidosis-evoked pain by targeting TRPA1 have not been met with success in humans [70]. Ischemia leads to increase in ROS generation which results in the formation of reactive carbonyl species like 4-hydroxynonenal (4-HNE) and 4-oxononenal (4-ONE), which act directly on TRPA1 [71, 72].

Recent studies suggest that TRPA1 may also be expressed in non-neuronal tissues. The strongest evidence for non-neuronal expression was provided by studies on intestinal enterochromaffin cells that respond to TRPA1 agonist with the release of serotonin that, in turn, activates underlying sensory nerve endings, a mechanism regulating gastrointestinal motility [73, 74]. TRPA1 expression was also reported in some permanent cell lines, including mast cell-, fibroblast and epithelial lines. Quantitative PCR studies have shown that neuronal transcript levels are at least several orders of magnitude higher than transcript levels in extra-neuronal tissues, making validation of extra-neuronal expression in other organs difficult [75]. Tissue detection of TRPA1 is also hampered by the poor specificity and validation of the available antibodies and the non-specific effects of the chemically reactive TRPA1 agonists. Recent studies using strategies to specifically ablate TRPA1 expression in sensory neurons recapitulated most of the findings from global TRPA1-deficient mice, including absence of acute and inflammatory pain modalities and hypersensitivity responses, suggesting that neuronal TRPA1 accounts for

most of the TRPA1-dependent physiological mechanisms [51, 52, 76]. However, as in the case of enterochromaffin cells, expression of TRPA1 in small cell populations may be difficult to detect. Additional sites with reported TRPA1 expression are melanocytes, odontoblasts, and insulin-producing β-cells of the Langerhans islets in the pancreas, and vascular endothelial cells. Via their endothelial expression, TRPA1 might regulate vascular tone, also might play a role in development of atherosclerotic disease [77].

Trigeminally-mediated pain involving TRPA1 channels could be relevant for the pathogenesis of headaches, especially migraine pain. TRPA1 channels have been shown to signal in response to a plant-derived headache- and migraine-inducing volatile compound, umbellone, emanating from the “headache tree”, *Umbellaria californica* [78, 79]. Since migraine is thought to occur when meningeal trigeminal nerves are activated, intraganglionic signaling between nasal airway-innervating and meningeal trigeminal branches would be a prerequisite [80]. Such a mechanisms was proposed for other environmental irritants that trigger headaches, including acrolein, the TRPA1 agonist in smoke and air pollution. Extended inhalational exposures of mice to acrolein were shown to increase meningeal vascular blood flow, supporting such a mechanism [81, 82]. A A common pro-migraine pathogenic mechanism is the release of calcitonin gene-related peptide (CGRP) from primary trigeminal sensory neurons, known to be triggered subsequent to TRPA1 activation [83]. CGRP is a strong meningeal vasodilator increasing blood flow and a target of novel antimigraine treatment successfully tested in clinical trials. Several clinically-effective antimigraine medicines, including herbal compounds such as petasin and parthenolides have been shown to modulate TRPA1 channels, implicating

TRPA1 as an important contributor to migraine and headaches, and as an additional target for innovative migraine drug development [83-86].

The pro-inflammatory actions of TRPA1 extend beyond pain. Recent studies suggest that TRPA1 is involved in inflammatory pathologies of the respiratory system, including allergic and chemically induced asthma and smoking-induced inflammation [37, 87-89]. TRPA1 inhibitors, or deletion of the TRPA1 gene, strongly reduced asthmatic airway contractions and pulmonary inflammation in mice and rats allergic to the experimental allergen, ovalbumin [87, 89].

Animal studies have revealed that the combined role of TRPA1 as a pain transducer and regulator of inflammation extends to many other inflammatory pain conditions, including arthritis and gout [90-93]. The gastrointestinal tract is also innervated by TRPA1-expressing nerve fibers. Here, TRPA1 plays a prominent role in acute mechanical distension pain of the colon and in gastrointestinal inflammatory pain and hypersensitivity during pancreatitis and in esophagitis due to chemical injury or allergic sensitization [94-105]. A recent study of colonic pain mechanisms reported that capsazepine desensitized colonic afferents after induction of colonic inflammation, yet this process was surprisingly independent of *Trpv1*, and apparently functioned via desensitization of TRPA1-expression in colon-innervating sensory neurons. For visceral pain of colon and pancreas, there appears to be a particular synergy between TRPV4 and TRPA1, see also the section on TRPV4 below [106].

2.3. TRPA1 in an inherited episodic pain syndrome and other forms of pain

The discovery of a human gain-of-function mutation in *TRPA1*, has greatly validated TRPA1's role as a "pain-TRP". This missense mutation is associated with a familial episodic pain syndrome [107] which is characterized by the presence of severe pain in the upper body accompanied by breathing difficulty, tachycardia, sweating, generalized pallor and stiffness of the abdominal wall [107]. These painful episodes are more likely if a carrier of the mutation has experienced challenge through fasting, fatigue, and exposure to cold and/or physical stress. Up to this point, for the entire TRP superfamily of channels, this hereditary pain-syndrome caused by a gain-of-function point mutation in TRPA1 is the only TRP-channelopathy in humans associated exclusively with pain.

This mutation is located in the linker region that connects transmembrane segment 4 and 5, thus intra-cellularly, where substitution of the asparagine 855 by a serine (N855S) changes voltage dependency to more negative potentials, and thus increasing TRPA1 activity without modifying the affinity for some activator [107, 108].

Interestingly, with regards to the N855 position in TRPA1, it was shown to be involved in the inhibitory action of TRPA1 antagonist HC-030031 in species selectivity studies human vs frog TRPA1 [109]. This raises the question of the correlation between the mutated amino acid associated with human disease and the amino acid changes observed in frog TRPA1 and zebrafish TRPA1b, both of which show insensitivity to HC-030031. Evolutionary comparison and these structure-activity-pharmacology studies suggest that N855 in huTRPA1 is a potentially high-priority target for developing effective TRPA1-modulating compounds.

The study reporting the N855 mutation in inherited episodic pain syndrome also observed that patients experienced breathing difficulties during painful episodes [107]. While no further details were provided, this observation suggests that TRPA1 may be involved in human respiratory control and, potentially, in asthmatic responses. This function was lent further support by a large longitudinal study that found several polymorphisms in the TRPA1 gene to be associated with childhood asthma [110].

Adding further weight to the concept that TRPA1 is a pain-TRP in humans, the human *TRPA1* gene was found to have methylation differences in identical twins that had different levels of pain sensitivity [111]. Increased expression of *TRPA1* was observed in the skin. This is consistent with an attenuating effect of DNA methylation on the *TRPA1* promoter affecting *TRPA1* gene expression.

We are optimistic that more wide-spread application of human DNA sequencing, especially in patients suffering from various forms of chronic pain and with their pain affecting multiple generations, will uncover additional polymorphisms and mutations in pain-TRPs such as TRPA1, that co-contribute to pathological pain [111].

Two non-hereditary forms of specific pain stand out in which TRPA1 has been found involved, the challenging-to-manage pai[90, 91, 112-119]n of sickle cell disease [120, 121], and the large unmet medical need of arthritis pain .

2.5 TRPA1 in itch

Itch is defined as an unpleasant sensation that evokes the desire/reflex to scratch. Itch can be classified as either acute or chronic. Acute itch serves an important protective

function as a sentinel against potentially harmful external agents such as insects, toxic plants, and other irritants, while at the same time, via the scratching component, removing the offending agent. On the other hand, chronic itch accompanies a number of skin diseases such as atopic dermatitis and Morbus Duhring, and systemic conditions, including hepatic cholestasis, diabetic neuropathy, kidney failure and lymphomas. Itch-sensitive neurons are divided into mechano-insensitive and mechano-sensitive C-fibers activated by pruritogens [122].

TRPA1 has been suggested to mediate histamine-independent itch in response to exogenous pruritogens such as chloroquine and cowhage spicules, and to endogenouspruritic mediators produced in the skin and other organs, includingbile acid, serotonin, lreactive oxygen species, leukotriene-B4, thymic stromal lymphopoietin (TSLP), and interleukins IL-13, IL-22, IL-31, and L-33 [3, 123-128].

Loss- and gain-of-function studies in mice have demonstrated that the G-protein coupled receptor, MrgprA3, is required for chloroquine-responsiveness in mice [129]. TRPA1 was found to signal down-stream of MrgprA3 in cultured sensory neurons and heterologous cells [125].

Endothelin (ET-1) can also evoke itch. It is also known as a vasoconstricting peptide that can stimulate nociceptors, pruriceptors and sensitizes them to noxious and pruritic stimuli. ET-1 has been implicated in both pain and itch via TRPA1, TRPV1 and TRPV4 [130-135].

Serotonin induced itch has been shown to be mediated via activation of serotonergic receptor HTR7 which signals to TRPA1 [136], but see also subsection “Role of TRPV4 in itch”, below. Aberrant serotonin signaling has long been linked to a variety of human chronic itch conditions, including atopic dermatitis. In a mouse model of this highly prevalent condition (10% of US Americans and in Europe), mice lacking HTR7 or TRPA1

displayed reduced scratching and skin lesion severity [136]. These data highlight a role for HTR7 and TRPA1 in acute and chronic itch, and suggest their antagonists may be useful for treating a variety of pathological itch conditions.Recent studies also show that TRPA1 contributes to pruritus due to allergic contact dermatitis, including contact dermatitis elicited by the poison ivy allergen, urushiol [75, 126].

Similarly, itch associated with severe liver disease, known as cholestatic itch, appears TRPA1-dependent. Bile acids induce itch in mice by activating bile-acid receptor TGR5 and TRPA1. TRPA1 acts downstream of G protein-coupled bile receptor such as TGR5. Antagonists of TGR5 and TRPA1, or inhibitors of the signaling mechanism by which TGR5 activates TRPA1, might be developed for treatment of cholestatic itch [137, 138]. However, serum levels of bile acids do not correlate with presence and intensity of cholestatic itch in liver patients, whereas the bioreactive phospholipid LPA does. Of note, LPA also signals to pruriceptor neurons via TRPA1 [123].

Again, TRPA1 not only serves as a sensor for pruritogens, but is also essential for maintaining skin inflammation, as shown in models of contact dermatitis and atopic dermatitis in which treatment with TRPA1 inhibitors reduced skin swelling, epidermal water loss and leukocyte infiltration [75, 127, 139].

In terms of development of TRPA1 inhibitory/modulatory compounds for human clinical use, as of today, TRPA1 inhibitors have a shared feature that all of them can be improved toward human studies [140, 141].

At a basic science level, on the other hand, elegant work has been conducted. TRPA1 has been found conserved along the evolutionary tree, with involvement of TRPA1 orthologues in pain-related behavior and inflammation in invertebrate model organisms [56, 142-159]

3. TRPM subfamily

The TRP melastatin subfamily (TRPM) consists of eight members, three of them associated with pain (TRPM2, TRPM3 and TRPM8). These ion channels resemble the overall molecular topology of the other TRP family members, although a major difference is the lack of ankyrin repeats in their amino end. We will focus on TRPM8 here and highlight key-references for M2 and M3.

3.1. TRPM2 pro-pain role

The thermo-TRP TRPM2 plays a role in pain signaling as recently discovered [54, 160-168].

3.2. TRPM3 pro-pain role

This is also true for the pregnonolone-receptor, TRPM3 [169-172].

3.3. TRPM8 in cold hypersensitivity and neuropathic pain

Among the TRPM members in the pain pathway, TRPM8 ion channels have been researched most in-depth. TRPM8 expression is restricted to a subset of small diameter sensory neurons of the trigeminal and dorsal root ganglia [173-175]. This channel is activated by cool and (noxious) cold temperatures between 10-23°C, and by natural compounds that evoke a cold-sensation such as menthol and eucalyptol (Figure 2) [173, 174, 176]. Similar to TRPV1 and TRPA1, TRPM8 activation evokes influx of calcium into the primary sensory neuron leading to its activation and propagation of action potentials. The role of TRPM8 in cold thermosensation has been widely explored *in vivo* in Trpm8-/- mice. These mice show a lack of response to cold thermal stimuli and chemical compounds that cause cold-

sensation such as icilin, and also lack of response to acetone-evoked evaporative cooling [176-178]. TRPM8 is also involved in orofacial and visceral (colonic) pain [47, 75, 179-181].

Nerve injury can result in cold allodynia, and it has been suggested that TRPM8 channels are a key substrate thereof [179, 182]. Rats subjected to constriction nerve injury show enhancement of withdrawal reflexes in response to evaporative cooling with acetone. They also have an increase in the number of neurons immunoreactive to the TRPM8 channel and increase in the number of neurons that are stimulated by the presence of menthol and cold temperatures [179].

TRPM8 has been linked with cold hypersensitivity mechanistically [182]. Unlike heat hyperalgesia which is produced by several algogenic agents, the TRPM8 cold sensitization appears evoked by select neurotrophic factors such as artemin and NFG which produce their effects through specific receptors co-expressed with TRPM8 in a subset of sensory neurons [183, 184]. This concept was convincingly demonstrated through the evaporative cooling assay, where neurotrophic factors were injected into the foot-pad, and subsequent application of acetone evoked evaporative cooling of the paw. Wild type mice showed an increase in their cold-response with growth factors, Trpm8-/- did not [183, 184].

TRPM8 ion channels' sensitizing effects on cold pain can be modulated by endogenous lipids. For example, TRPM8 activation by cold temperatures and menthol requires the presence of PIP_2 (a membrane phospholipid), which interacts directly with positively charged amino acids located in the TRP box of the channel [185]. Furthermore, PIP_2 by itself can activate the channel [185]. Therefore, TRPM8-mediated cold allodynia and hypersensitivity could be attenuated by regulating PIP_2 levels such as via activation of

calcium-dependent PLC which hydrolyzes then PIP_2 to produce DAG and IP_3 [185]. Moreover, DAG can activate the PKC pathway which then attenuates TRPM8 gating. This effect is mediated by specific TRPM8 serin-phosphorylation events [186].

In addition, TRPM8 activity is endogenously regulated by the $iPLA_2$ pathway, a calcium-insensitive phospholipase which produces lysophospholipids and free polyunsaturated fatty acids (PUFA) from the hydrolysis of sn-2-glycerophospholipids [187]. Interestingly, inhibition of $iPLA_2$ attenuates TRPM8 currents evoked by cold, menthol and icilin [187]. Moreover, lysophosphatidylcholine (LPC) released from the $iPLA_2$ pathway can activate TRPM8 even at 37°C [187]. Furthermore, LPC evokes cold hypersensitivity in a TRPM8-dependent manner since this behavior was absent in Trpm8-/- mouse [188].

Pointing to endogenous regulatory mechanisms, PUFAS, such as arachidonic, eicosapentaenoic and docosahexaenoic acids, which also are released by iPLA2, are endogenous inhibitors of TRPM8 [187].

Therefore, the $iPLA_2$ pathway can produce contrasting effects on TRPM8 activation: an activating effect by lysophospholipids and a modulating-inhibitory effect by PUFAS. Is one pathway “dominant” over the other ? - Equimolar application of LPC and arachidonic acid favors the activation of TRPM8 by LPC, possibly implicating this mechanism in cold pain and cold hypersensitivity phenomena [187].

Selective TRPM8 inhibitors were effective in reducing cold sensing and also cold pain, suggesting that TRPM8 contributes to both innocuous and noxious thermal sensing [47, 189]. Another study using a TRPM8-specific inhibitor reported no significant effects on

neuropathic mechanical allodynia, however, effects of cold allodynia were not investigated [190].

While TRPM8 inhibition may be effective in reducing neuropathic cold pain, TRPM8 agonists have long been known to have analgesic properties. Menthol is widely used as a topical analgesic and inhaled antitussive with respiratory counterirritant properties. Menthol, and TRPM8-selective menthol analogs, were shown to suppress nocifensive responses to several acute pain stimuli, including capsaicin, acrolein and acid [191]. The analgesic effects of menthol were absent in TRPM8-deficient mice [191]. Menthol also suppressed inflammatory pain in a TRPM8-dependent manner. [191]. Icilin was demonstrated to diminish colitis-associated pain in a TRPM8-dependent manner [192]. Eucalyptol, the TRPM8 agonist in eucalyptus oil, also suppressed pain and respiratory inflammation and irritation [181, 193]. TRPM8 selective agonists may have improved analgesic properties compared to menthol that can cause irritation in some patients [191].

Possibly bespeaking of a role for TRPM8 in controlling itch, menthol has been reported to function as an anti-pruritic in therapy-resistant pruritus in lichen amyloidosis [194] and hydroxyethyl starch-induced itch [195] but these clinical observations leave open the possibility of menthol also acting on TRPA1 that is also sensitive to menthol. A more recent study demonstrated that menthol's anti-pruritic actions depend on inhibitory interneurons in the spinal cord that receive input from menthol-sensitive fibers and dampen input from pruriceptors [196].

4. TRPV Subfamily

The TRP vanilloid subfamily is named after its founding member, the TRPV1 channel, which is activated by the classic natural pungent vanilloid, capsaicin. Mammalian TRPV1 channel in this subfamily sensitive to vanilloids compounds. The other members share amino-acid sequence homology. This subfamily consists of six members (TRPV1-TRPV6), all of them are cation non-selective channels where TRPV1/3/4 show a preference for Ca^{2+} and they are overall linked to pain and itch, thus they will be considered in this section in some more detail.

4.1. TRPV1 is a widely studied portal to pain

The TRPV1 channel is a representative member of the TRPV subfamily and is the most well-characterized TRP channel. TRPV1 can be activated by various stimuli, including temperature (~42°C), pH, and a wide range of both endogenous and exogenous compounds (Figure 3). Its main exogenous ligand is capsaicin, a compound from pungent peppers that activates the channel. The beneficial effects of capsaicin on nociception were identified before the TRPV1 channel was discovered [197]. TRPV1 was cloned in 1997 from a cDNA library isolated from capsaicin and temperature-stimulated nociceptor neurons [198]. Subsequent characterization of TRPV1 has revealed its key role in nociception. In addition, with the use of Trpv1-/- mice, TRPV1 has been shown to be important in pain sensation [199]. Due to its importance in these physiological processes, several compounds have been developed to modulate the activity of TRPV1 to eliminate or reduce pain.

4.1.1. Expression of TRPV1 in the nervous system

TRPV1 is preferentially expressed in sensory neurons of the PNS. In the PNS, TRPV1 is primarily expressed by the small and medium nociceptor neurons of the dorsal root ganglion (DRG), trigeminal ganglion (TG), nodal ganglion (NG), and sympathetic ganglion (SG), in the peptidergic and non-peptidergic C fibers, and in some Aδ fibers [200-202]. In addition, it is expressed at lower levels in nerve fibers that innervate the bladder [203], lungs [204, 205], and cochlea [206], as well as in the upper respiratory tract, where its function is to sense irritant compounds [207, 208].

TRPV1 is expressed in several regions of the CNS, specifically in laminae I and II of the dorsal horn of the spinal cord, where it modulates the synaptic transmission of nociceptive signals from the periphery [209]. TRPV1 is primarily expressed in the presynapse, though there have been studies that demonstrated its postsynaptic expression. However, its postsynaptic expression has not been shown to be related to nociception [210]. The use of transgenic mice expressing reporter proteins driven by the TRPV1 promoter has revealed the areas in which TRPV1 is expressed [211]. Expression has been observed in the nerve fibers of small and medium diameter nociceptor neurons in the cornea, bladder, and skin; in the dorsal root ganglia and trigeminal ganglia in the dorsal horn of the spinal cord; and in some regions of the brain, such as the brain stem, the nucleus caudalis, the nucleus ambiguus, the olfactory bulb, and the nucleus parabrachialis [211]. Interestingly, TRPV1 – non-capsaicin receptor channels, thus TRPV1 splice-variant transcripts – have been cloned from supra-optic microdissection. These channels appear to function in organismal response to systemic hypertonic osmolality and to organismal body temperature [212].

4.1.2. TRPV1 in inflammatory pain

When a skin lesion is produced, a wide variety of proinflammatory molecules are released, such as bradykinin, prostaglandins, leukotrienes, serotonin, histamine, substance P, thromboxanes, platelet-activating factor, adenosine and ATP, protons, and free radicals. During inflammation, cytokines, such as interleukins (IL), tumor necrosis factor, and neurotrophins, particularly NGF, are also generated. In general, these mediators sensitize TRPV1, increasing the probability of a stimulus activating TRPV1. The first evidence of the role of TRPV1 in peripheral pain processing was generated by observations made in mice injected with the TRPV1 antagonist capsazepine. Capsazepine injection attenuated inflammation-induced thermal hyperalgesia [199, 213] (however, also note above comments on selectivity of capsazepine – possibly TRPA1 desensitizing effects). $TRPV1^{-/-}$ mice do not respond to noxious temperatures and present with an attenuation of inflammation-induced thermal hyperalgesia. Additionally, $TRPV1^{-/-}$ mice do not experience thermal hyperalgesia after CFA (Complete Freund's Adjuvant) administration.

When bradykinin is released, it acts on β2 receptors, which in turn activate PKC to facilitate phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis by phospholipase C to produce inositol 3-phospate and DAG (diacylglycerol), two compounds that can affect TRPV1 function [214]. DAG is a TRPV1 agonist that interacts with tyrosine 511 of its S3 domain [215]. PIP_2 binds to the C-terminal end to positively regulate TRPV1 [216]. Prostaglandins increase capsaicin-induced currents in DRG neurons and reduce the temperature activation threshold of the channel. These effects have been corroborated in Trpv1-/- mice [217]. TRPV1 integrates multiple proinflammatory stimuli, and for this reason, being able to modulate this pathway appears a desirable goal. For example, as a natural

compound, oleic acid has recently been shown to inhibit TRPV1 activation, thus modulating pain and itch [218].

4.1.3. TRPV1 in neuropathic pain

Neuropathic pain can occur when there is an injury or dysfunction of the CNS or PNS and is a condition that is difficult to treat. The TRPV1 channel is a polymodal nociceptor, and it is modulated by proinflammatory mediators. The process of sensitization increases excitability, causing hyperalgesia and allodynia. However, there is contradictory evidence regarding the role of TRPV1 in neuropathic pain. It has been found that in TRPV1$^{-/-}$ mice, there is no change in pain-related behavior after nerve damage [199, 219]. In line with this observation, pharmacological TRPV1 inhibition decreases pain in several animal models of neuropathic pain [164, 220]. Interestingly, an increase in TRPV1 expression has been found in several models of neuropathic pain, such as the spinal nerve ligation model. Increased TRPV1 expression correlates with the development and maintenance of thermal hyperalgesia [221]. Diabetic neuropathy models have difficulties in mimicking the clinical reality of this dreaded complication, and implications of TRPV1 and TRPA1 have been postulated here, but await an improvement of these models or translational findings in humans so that validity of the results can be increased[222-224]. Finally, it has been found that TRPV1 plays an important role in cancer-induced chronic pain. In bone cancer, TRPV1 is overexpressed in DRG neurons, and this increased expression correlates with neuropathic pain [225]. In addition, it has also been shown that LPA activates TRPV1 through a direct interaction of a lysine at position 710 located in the C-terminus of the channel which produces acute pain in animals injected with the lipid. Pointing to the key role of TRPV1, this response is attenuated in Trpv1-/- mice [226].

4.1.4. TRPV1 in visceral pain

TRPV1 is expressed in DRG neurons that innervate the colon, pancreas, stomach, duodenum, and bladder and is responsible for pain produced by gastrointestinal (chronic) inflammation [227]. In tissue biopsies of patients with inflammatory bowel disease (IBD), an increase in TRPV1 expression has been observed, as in patients with rectal hypersensitivity. Notably, increased channel levels correlate with hypersensitivity levels [228]. The administration of acetic acid and capsaicin increases the activity of afferent fibers in the pelvis in control mice and in mice with DSS-generated colitis (dextran sulfate sodium-generated colitis). However, the response to capsaicin in DSS-treated mice was greater than in controls [229]. Interestingly, Trpv1-/- mice were less sensitive to these treatments, highlighting the role of TRPV1 in this type of pain [230].

4.1.5 TRPV1 in itch

TRPV1 is involved in both acute and chronic itch conditions [231]. For example TPRV1 has been suggested to be involved in many chronic itch conditions like rosacea [232], atopic dermatitis [233], and prurigo nodularis, a skin condition characterized by itchy nodules on arms and legs [234]. However, as an initial cautionary note, in humans, treatment with a TRPV1 inhibitor did not improve chronic itch in patients [235].

Histamine is released from mast cells when tissues are inflamed or stimulated by allergens. Histamine excites sensory neurons by activating TRPV1 and this activation is mediated by the production of 12-HPETE, a downstream metabolite of PLA_2 and LO [236]. In cultured sensory neurons, histamine evoked inward currents that were reduced by capsazepine, a TRPV1 blocker, or when histamine receptor subtype 1 (H1R) and TRPV1 were expressed heterologously, but not when H1R or TRPV1 were expressed separately.

In addition, histamine caused Ca^{2+} influx into sensory neurons in wild-type mice but not in Trpv1-/- mice. When injected subcutaneously into the necks of mice, histamine caused bouts of scratching, which were greatly reduced by pretreatment with capsazepine, and by inhibitors of PLA_2, LO, and H1R and in Trpv1-/- mice. These results suggest that TRPV1 might play a role in mediating the pruritogenic action of histamine via the PLA_2/LO pathway [237].

IL-31RA is a functional receptor expressed by a small subpopulation of neurons positive for IL-31RA, TRPV1 and TRPA1 and is a critical neuroimmune link between TH2 cells and sensory nerves for the generation of T cell-mediated itch [124]. Cutaneous and intrathecal injections of IL-31 evoked intense itch, and its concentrations increased significantly in murine dermatitis skin. Both human and mouse dorsal root ganglia neurons express IL-31RA, largely in neurons that co-express TRPV1. IL-31-induced itch was significantly reduced in Trpv1-/- and Trpa1-/- mice [124].

Toll-like receptors (TLRs) are expressed in immune cells to regulate innate immunity. TLR 3, 4, and 7 are expressed in dorsal root ganglia neurons that co-express TRPV1, gastrin-releasing peptide (GRP), and MrgprA3 [238] [239]. Topical application of Imiquimod, a TLR7 activator and a clinically used anti-viral and anti-tumor drug used to treat genital warts, often causes itch as a side effect. TLR7 activation by imiquimod induced scratching and also generated inward currents and action potentials in DRG neurons [238]. Signaling mechanisms that link TLRs / TLR7 with TRPV1 need to be elucidated.

4.2 TRPV3 in pain and itch

A member of the TRP vanilloid subfamily important for temperature detection is the TRPV3 channel. This non-selective cation channel is abundantly expressed in skin

(specifically in keratinocytes) [240] and has been also detected in neurons from dorsal root and trigeminal ganglion [241] . TRPV3 protein has 43 % identity to TRPV1 and is a warn temperature sensitive but capsaicin and low pH insensitivity ion channel [240, 241].

Among the ligands for TRPV3 activation are plant-derived compounds such as camphor, carvacrol and thymol [242], furthermore, TRPV3 activity is enhanced by some endogenously produced compounds such as arachidonic acid which is an unsaturated fatty acid released during inflammation [243]. Unlike TRPV1 activation which requires the metabolism of this fatty acid to evoke currents, the enhancement of TRPV3 function is independent of arachidonic acid oxidation [243]. It is possible that in some skin pathologies as psoriasis where arachidonic acid levels are elevated [244], TRPV3 activation could contribute to chronic dermatitis.

Another TRPV3 agonist is farnesyl pyrophosphate (FPP), an endogenously produced intermediate metabolite of the mevalonate pathway [245] which produces isoprenoids which can also activate TRPV3. FPP was identified as novel pain-producing compound through specific TRPV3 activation [246].

4.2.1 TRPV3 in nociceptive and inflammatory pain

Although, the physiological role of TRPV3 is still unclear, some studies suggest that this ion channel can contribute to pain/nociception. For example, mice overexpressing TRPV3 in keratinocytes release prostaglandin E2 and produce thermal nociception and hyperalgesia [247]. Contrary, Trpv3-/- mice have an impaired response to warm and also noxious-warm temperatures [248].

Finally, some endogenous antagonist to TRPV3 activation has been isolated, for example, some compounds synthetized from the omega-3 fatty acids such as the resolvins are linked to anti-inflammatory and anti-nociceptive effects. One of them, 17(R)-resolvin D1 is a specific inhibitor of TRPV3 [26]. This resolvin affects the voltage-dependence of TRPV3 thus decreasing the channel's activation [26]. The inhibitory effects of this resolvin are specific through TRPV3 inhibition since the pain behavior produced by capsaicin or cinnamaldehyde are unaffected. However, nociceptive behavior produced by intradermal injection of FPP, a selective activator of TRPV3, is attenuated by co-injection with resolvin D1 [26].

4.2.2 TRPV3 in itch

A missense mutation in TRPV3, Gly573Ser has been found in individuals with Olmsted syndrome (OS), a rare congenital disorder, with severe itching, peripheral pain, and palmo-plantar hyperkeratosis among other symptoms [249]. In mice, TRPV3^{Gly573Ser} led to increased ion channel activity in keratinocytes and was associated with hairlessness, also a liability to develop severe dermatitis in DS-*Nh* mice [250]. Also, TRPV3^{Gly573Ser} induced a higher nerve growth factor response to heat and increased scratching behavior. In transfected HEK293 cells expressing TRPV3^{Gly573Ser}, increased inward currents were recorded, defining TRPV3^{Gly573Ser} as a gain-of-function. This might lead to increased likelihood of apoptosis of keratinocytes and keratinocyte hyperproliferation via Ca++ signaling, explaining the hyperkeratosis phenotype in affected individuals [251]. Taken together, these observation in a human TRPV3 gain-of-function channelopathy suggest that TRPV3 channels are regulating skin keratinocytes' activity and function, and that TRPV3 over-activity in these cells can evoke not only over-proliferation and

hyperkeratosis, but possibly also sensory phenomena, namely pathologic itching and irritation/pain. However, more in-depth reverse-translation will be needed to tether apart the contributions of keratinocyte TRPV3 and sensory neuron TRPV3 in OS, and in general.

4.3 TRPV4 functions as pain-TRP and itch-TRP

As a member of the TRPV subfamily, TRPV4 is a multi-modally activated, nonselective cation channel. Functional expression of TRPV4 has been detected in nerve cells and non-neuronal neural cells [252]. Importantly for this chapter, one of the initial discovery papers lays out a rationale how TRPV4 can function in pain (see Fig. 4) by demonstrating its expression in trigeminal ganglion (TG) sensory neurons, in small-diameter neurons, and reasoning about a role for TRPV4 in pain signaling [252]. This initial conjecture has now been confirmed, e.g. a search for “TRPV4 pain” generated 185 references, out of a total of 1085 references for “TRPV4”. Primary sensory neurons with TRPV4 expression include pain-sensing neurons in dorsal root ganglion (DRG) [7, 253] [254] and TG [255, 256] [96, 257], most recently also satellite cells in sensory ganglia [258], in the CNS for pain transmission in astrocytes [259], microglial cells [260] and neurons [261, 262] [263] . Outside the nervous system, TRPV4 has been found in innervated cells such as prominently in chondrocytes [264], vascular endothelia [265] and innervated epithelia such as skin keratinocytes [266, 267], airway epithelial cells [26, 268], colonic epithelia [269] and odontoblasts [270]. Co-labeling experiments with neuronal markers and size determination revealed that TRPV4-expressing sensory neurons are rather nociceptive [133, 271, 272], a finding in keeping with evidence that suggests that TRPV4 is involved in nociception both physiologically

and in sensitized states such as inflammation and nerve injury [7, 271]. Of note, TRPV4 expression in trigeminal ganglia appears increased over that in DRGs, up to this day not fully explained and how this difference correlates with sensory functions [273, 274].

4.3.1. TRPV4 in transduction of mechanically-evoked pain

Trpv4-/- animals, generated after the initial description, were found to have defective mechanosensation in the physiologic, non-sensitized state [255 , 273], bespeaking of a role for TRPV4 in non-sensitized mechano-transduction. This finding was supported and extended by assessing function of mammalian TRPV4 in ASH head nociceptor neurons of *C. elegans* in a mutant line of animals which were lacking the proto-ancestral osmo-mechano-TRPV channel, OSM-9 [275] , see also [5] .

4.3.2. TRPV4 in inflammatory pain

TRPV4 in sensory neurons can be sensitized by pro-inflammatory mediators, such as prostaglandin E2, activator of proteinase-receptor 2 (PAR-2), an integrator of proteolytic signaling in inflammation, especially allergic inflammation, histamine, and/or serotonin, leading to increased nociception to hypotonic, mild hypertonic stimuli or mechanical stimuli [271, 276, 277]. Grant et al. found that TRPV4 was co-expressed by rat DRG neurons with proteinase-activated receptor 2 (PAR2), so that intraplantar injection of PAR2 agonist caused mechanical hyperalgesia in mice and sensitized pain responses in a TRPV4-dependent manner, e.g. to the TRPV4 activator 4α-PDD and also hypotonic solutions. Deletion of Trpv4 prevented PAR2 agonist-induced mechanical hyperalgesia and sensitization [7]. Further studies demonstrated that cathepsin-S [138] or elastase-mediated activation of PAR-2 [278] activated TRPV4 and sensitized nociceptors to function in a

hypersensitive manner resulting in inflammation and pain. In the lab of the senior author, we established a key role for TRPV4 ion channels in the pain response to TMJ inflammation. In Trpv4$^{-/-}$ mice with TMJ-inflammation, attenuation of bite force, a surrogate of TMJ-injury mediated pain in humans and validly reverse-translated into mice, was significantly and robustly reduced vs WT mice. Furthermore, TRPV4 protein expression in the TG was dramatically upregulated after TMJ inflammation, in synchrony with clinical severity after TMJ inflammatory injury, suggesting TRPV4 expression in the TG as a critical locale for behavioral sensitization [96]. In favor of this concept, TRPV4 was expressed in TMJ-innervating sensory neurons, and TRPV4-expressing TG sensory neurons co-expressed CGRP and phosphorylated ERK, a biochemical signaling activation marker in response to injury. pERK-TRPV4 co-expressing TG sensory neurons became more numerous in response to TMJ inflammatory injury, suggesting that TRPV4-mediated Ca^{2+} influx into TG sensory neurons evokes MAPK activation in these neurons, which functions as a molecular substrate of the sensitization response after inflammatory injury. At least as interesting was our finding that peripheral injury to the joint after inflammatory injury was independent of the genotype, whereas the pain response was strikingly dependent on Trpv4 or ability of the animal to generate phosphorylated ERK in their neurons. In another study by Denadai-Souza et al., TRPV4 expression in the TG was demonstrated, in addition in TMJ synovial cells [119, 279]. Taken together, it emerges that TRPV4 plays an important role in inflammatory joint pain of the TMJ, perhaps beyond that, rather at the level of joint-innervating sensory neuron than peripheral TRPV4-expressing cells, and that therefore TRPV4 becomes an attractive therapeutic target to address TMJD, possibly additional forms of joint pain, a matter of increasing unmet medical need, given the fact that TMJD is a prevalent craniofacial pain disorder, and given

the increase in prevalence of age- and obesity-associated osteo-arthritis and post-traumatic osteo-arthritis [280].

TRPV4's role in facilitating and promoting inflammation and pain was also supported by observations in a mouse model of skin inflammation in which UVB radiation generated sunburn tissue damage (UV-burn) and associated pathological pain. Following UVB over-exposure of their hindpaws, an area of mouse skin with increased resemblance to human skin, mice with induced Trpv4 deletions in keratinocytes, also mice treated with topical TRPV4-inhibitors to their hindpaws, became virtually resistant to noxious thermal and mechanical stimuli vs. control animals, also showed dramatically reduced skin inflammation. These findings indicate that TRPV4-expressing keratinocytes of the hindpaw can "moonlight" as non-neural sensing cells and pain-generating cells. In other words, activation of TRPV4 channels in skin keratinocytes, not in innervating sensory neurons, suffices to switch on neural pathological-pain circuits and response-mechanisms. Importantly, we demonstrate a form of non-neural phototransduction in response to UVB radiation in skin keratinocytes, completely dependent on TRPV4. Exploring a possible underlying mechanism, we found that epidermal keratinocyte TRPV4 is essential for UVB-evoked skin tissue damage and increased expression of the pro-pain (also pro-itch !) mediator endothelin-1 (ET-1) [266]. Importantly, we also recorded an important negative finding which will become the starting point for relevant future studies, namely that keratinocyte-derived ET-1, dependent on TRPV4 function and UVB-mediated activation in these cells, was not the algogenic signal to innervating peripheral nerve projections.

4.3.3. TRPV4 in neuropathic pain

TRPV4 has been implicated in nerve pain in several preclinical rodent pain models, such as paclitaxel-induced neural injury leading to painful peripheral neuropathy (CIPN) and chronic mechanical compression-injury of the DRG [50, 281, 282]. Cerebrospinal fluid administration of antisense oligodeoxynucleotides specific for TRPV4, which reduced the expression of TRPV4 in sensory neurons, attenuated paclitaxel-induced mechanical hyperalgesia as well as hyperalgesia caused by hypotonicity [281]. TRPV4 appears co-expressed with TRPC1 and TRPC6 in DRG neurons, and it has been proposed that TRPC1 and TRPC6 may act in concert with TRPV4 to mediate mechanical hyperalgesia induced by paclitaxel and cis-platin [282], possibly representing a more general mechanism of pain in CIPN. In addition, paclitaxel can stimulate the release of mast cell tryptase, which activates PAR2 and, subsequently, protein kinases A and C, resulting in mechanical and thermal hypersensitivity through TRPV4 sensitization [50]. These results suggest that TRPV4 plays an important role in painful peripheral neuropathies caused by paclitaxel and related taxanes, affecting more than half of taxane-treated cancer patients. This is a large unmet medical need which not only translates to substantial patients' quality-of-life, but indeed to their ability to successfully undergo adjuvant chemotherapy and thus emerge with increased years of survival. We reach the same conclusion as for joint- or skin-mediated forms of pain and inflammation, as discussed above, namely that TRPV4 is a promising target for anti-pain therapy. To that end, we have recently developed novel TRPV4-inhibiting compounds in our group [263]. Two of our compounds also potently co-inhibited TRPA1 ion channels, which would be a highly beneficial combination for CIPN

pain, as well as for other forms of pain reviewed in some detail below, namely visceral pain of colon and pancreas and headaches [263]

TRPV4 is involved in mediating mechanical allodynia in preclinical models of chronic compression of the DRG (CCD model) [254]. Post-CCD, Trpv4 mRNA and protein expression were increased when compared with the sham group, with the highest level at 7 days post-CCD. Knock down of TRPV4 partly reversed the CCD-induced mechanical allodynia. CCD rats showed thermal hyperalgesia and increased nitrite production [283]. The thermal hyperalgesia was reduced by intrathecal blockers targeted at any site along the TRPV4-NO-cGMP-PKG pathway. Inhibition of TRPV4 and/or nitric oxide synthase (NOS) decreased the nitrite production in DRG in CCD rats. Changes in the level of nitrite was shown to positively associate with the changes of thermal hyperalgesia. Identified signaling pathways involved in the CCD neuropathic pain model include NF-kappa-B, which mediate the TRPV4-NO pathway [284] and the p38 pathway [285].

4.3.4. TRPV4 in visceral pain

Trpv4 mRNA is enriched in colon-innervating sensory neurons and TRPV4 protein colocalized in a subset of fibers with CGRP, the substrate of neurogenic inflammation in mice. Mechanosensory responses of colonic serosal and mesenteric afferents were enhanced by a TRPV4 agonist and dramatically reduced in Trpv4-/- mice. The behavioral responses to noxious colonic distention were also substantially reduced in mice lacking Trpv4 [286]. Similarly, another study found that TRPV4 agonist 4α-PDD specifically activated a cationic current and calcium influx in colon-innervating DRG neurons and caused a dose-dependent visceral hypersensitivity. TRPV4-targeted but not mismatched siRNA intra-thecal treatments were effective at reducing basal visceral nociception, and

TRPV4-activators 4α-PDD or PAR2 agonist induced hypersensitivity [253]. PAR2 exacerbated visceromotor responses, an indicator of mechanical hyperalgesia, which was absent in Trpv4-/- mice, indicating TRPV4 is required for PAR2-induced mechanical hyperalgesia in the colon, and excitation of colon-innervating sensory neurons [287]. A more recent study showed that both TRPV4 and TRPA1 mediated colonic distension pain and CGRP release. Unlike TRPV4 and TRPA1, the role of TRPM8 seems to be confined to signaling more extreme noxious distension [180]. Interestingly in this context, levels of the TRPV4 agonist 5,6-EET were increased in IBS colon biopsies. Increases correlated with pain and bloating scores. Small interfering RNA knockdown of TRPV4 in mouse primary afferent neurons inhibited the hypersensitivity caused by supernatants from IBS biopsies in mice. Polyunsaturated fatty acid metabolites extracted from IBS biopsies or colons of mice with visceral hypersensitivity activated mouse sensory neurons in culture via TRPV4 [288]. These data indicate that TRPV4 contributes to visceral pain, with relevance to human disease.

Of note, TRPV4 also plays an important role in pancreatitis pain, a large unmet clinical need in visceral pain. Immunoreactive TRPV4 was detected in pancreatic nerve fibers and in DRG neurons innervating the pancreas, which were identified by retrograde tracing. Activation of TRPV4 with 4α-PDD increased intracellular Ca^{2+} in these neurons in culture. The secretagogue cerulein induced chemical pancreatitis, c-Fos expression in spinal cord dorsal horn neurons of pancreas-innervating segments and pancreatitis pain behavior. All of these were suppressed in Trpv4-/- mice [225]. Using a chronic pancreatitis model induced by high-fat and alcohol diet (HFA), Zhang et al., demonstrated that TRPV4 expression was increased in pancreatic stellate cells (PSCs). Calcium signal of PSCs from HFA-fed

rats in response to 4α-PDD was dramatically higher than that of cells from control rats. Tumor necrosis factor-α (TNF-α) increased responses to TRPV4-activator 4α-PDD in control PSCs [289]. Furthermore, inhibition of TRPV4 with systemic injection of the selective blocker HC067047 effectively alleviated mechanical and thermal hypersensitivities of rats with HFA chronic pancreatitis in a dose-dependent manner [290]. In the lab of the senior author, we recently found that blockade of both TRPV4 and TRPA1 with the dual inhibitor, compound 16-8, injected intra-peritoneally, dramatically attenuated pancreas edema and inflammation induced by caerulein. Furthermore, serum amylase, a marker of inflammatory injury of the pancreas was also significantly reduced by 16-8 treatment. Perhaps most importantly, pancreatits pain behavior was virtually eliminated in response to compound 16-8 [263]. Using this well-established pre-clinical model, these studies provided evidence that dual channel inhibitors targeting TRPV4 and TRPA1 might be a rewarding new concept to meet the grave unmet clinical need of pancreatits pain.

4.3.5. TRPV4 in cranio-facial pain and headaches

Using in vitro patch-clamp electrophysiology of trigeminal neurons retrogradely labeled from the dura, around half of identified dural afferent neurons generated currents in response to hypotonic solutions and 4α-PDD, indicating robust dural innervation bia TRPV4. This is so relevant because of the known involvement of TRPV4 in mechanotransduction, and its sensitization to mechanical cues under inflammatory conditions. Activation of meningeal TPRV4 using hypotonic solution or 4α-PDD in vivo resulted in facial allodynia that was blocked by the TRPV4 antagonist RN1734 [291]. These data indicate that activation of TRPV4 within the meninges produces afferent nociceptive signaling from the head that may contribute to migraine headache. In the lab of the senior

author, we recently found TRPV4 to be important for trigeminal nocifensive behavior evoked by formalin whiskerpad injections. This conclusion is supported by studies with Trpv4$^{-/-}$ mice and TRPV4-specific antagonists GSK205 and HC067047 [267]. Our results suggest that TRPV4 acts as a relevant signaling molecule in irritation-evoked trigeminal pain, at the level of a functional formalin receptor in TG sensory neurons. TRPV4-antagonistic therapies can therefore be envisioned as novel analgesics, possibly for specific targeting of trigeminal pain disorders, including headaches, with a mandate to further elucidate pathophysiology but also to continue translational medical development.

Taken together, based on the role of TRPV4 in pain as currently understood, future studies are needed to address many important questions, a few of which are 1) how sensory-neuronal TRPV4 signals in a pain-relevant manner, particularly pathological pain, readily addressable via appropriate genetic targeting strategies; 2) possibly co-contributing role of glial cells-expressing TRPV4 [259], co-signaling with other pain-TRP channels, in particular TRPA1 given the observed overlap in TRPV4-TRPA1 function in several important and relevant forms of pain, such as visceral pain of the colon and pancreas, chemotherapy-induced peripheral neuropathy pain, especially mediated by taxane, headaches, formalin-evoked pain as important model, mechanically-evoked pain in general and also chronic cough as an upper airway equivalent of chronic pain [50, 57, 78, 180, 253, 257, 263, 286-288, 292].

4.3.6. TRPV4 in itch

The finding of the TRPV4-dependent secretion of the pruritogen, ET-1, by keratinocytes, led the senior author's group to question whether TRPV4 plays a role in itch, in particular whether TRPV4 in keratinocytes of the epidermis can drive scratching behavior [266]. To

address this question the focus was first on acute itch, and specifically to examine prototypic examples of histaminergic itch, including ET-1-evoked itch, plus chloroquine-caused non-histaminergic itch as control. In this study an exciting new function of TRPV4 in forefront signaling of the integument was reported, namely that TRPV4 in epidermal keratinocytes functions as a pruriceptor-TRP channel in acute histaminergic itch, including itch evoked by ET-1, not in non-histaminergic itch evoked by chloroquine. Direct activation of TRPV4 channels also evoked scratching behavior which appears completely dependent on TRPV4-expression in keratinocytes, thus underscoring the role of this cell and its expression of TRPV4 in itch [135]. Complementing findings in *Trpv4* keratinocyte-specific inducible knockout (*Trpv4* cKO) mice, Ca^{++} transients in response to histaminergic pruritogens in cultured primary keratinocytes were shown that depend on TRPV4. Ca^{++} influx via TRPV4 then up-regulates phosphorylation of the MAP-kinase ERK in keratinocytes. Consequently, topical transdermal treatment with a selective inhibitor of TRPV4 was effective as an anti-pruritogen. Moreover, we observed similar in-vivo anti-pruritic effects when topically targeting MEK, upstream of ERK, with a selective inhibitor.

Serotonin (5-hydroxytryptamine [5-HT]) induced itch has been shown associated with TRPV4 [168]. Approximately 90% of 5-HT-sensitive DRG neurons assessed by calcium imaging were immunoreactive for TRPV4. TRPV4 knockout mice exhibited significantly fewer 5-HT-evoked scratching bouts compared with wild-type mice. Pretreatment with a TRPV4 antagonist significantly attenuated 5-HT-evoked scratching in vivo. In cultured primary murine DRG neurons, the response of neurons after 5-HT application was attenuated in Trpv4-/- mice. A TRPV4 antagonist suppressed 5-HT-evoked responses in dorsal root ganglion cells from wild-type mice. These results indicate that 5-HT-

induced itch is linked to TRPV4. Extending on the role of TRPV4 in itch, TRPV4 signaling in some subgroups of DRG neurons was suggested to be facilitated by TRPV1 in itch. Both latter studies, on the role of serotonin in itch, and the suggested TRPV4/TRPA1 co-signaling, are interesting and push the barrier of knowledge, yet there are clear limitations by use of Trpv1-/- and Trpv4-/- pan-null animals as key tools, not cell-type specific and inducible genetically-engineered animals.

5. Concluding Remarks

Nociception is an important physiological process detecting harmful signals resulting in pain perception. In this chapter, we have reviewed some important experimental evidence involving some TRP ion channels as molecular sensors of chemical, thermal and mechanical noxious stimuli to evoke the pain sensation. Among them are the TRPA1 channel, members of the vanilloid subfamily (TRPV1, TRPV3 and TRPV4) and finally some members of the melastatin group (TRPM2, TRPM3 and TRPM8).

Given that pain is a pro-survival, evoluationarily honed protective mechanism, care has to be exercised when developing inhibitory/modulatory compounds targeting specific pain-TRPs so that physiologic protective mechanism are not disabled to a degree that stimulus-mediated injury can occur. Such events have impeded development of safe and effective TRPV1-modulating compounds and diverted substantial resources. A beneficial outcome can be readily accomplished via simple dosing strategies, and also by incorporation of medicinal chemistry design features during compound design and synthesis. Beyond clinical use, where compounds that target more than one channel might have a place,

possibly providing advantageous features, highly specific and high-potency compounds will be helpful in mechanistic discovery at a structure-function level.

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Author Contributions

All authors wrote the paper and approved the final version of the manuscript.

7. References

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8. Figure Legends

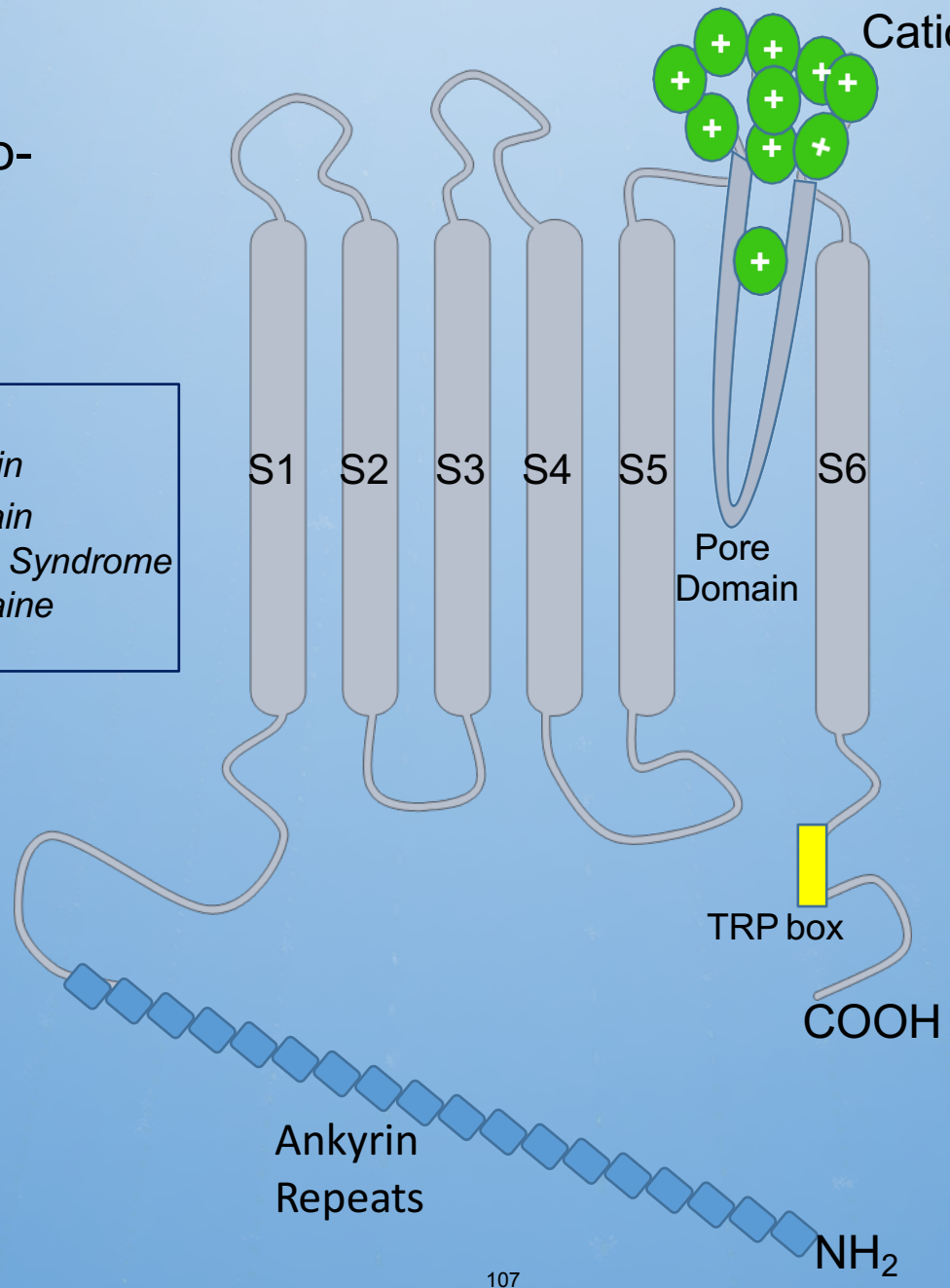
Figure 1. TRPA1 ion channel, schematic. Representative schematic of a subunit of TRPA1 channel showing the transmembrane segments (S1-S6), a pore domain former between S5-S6, several ankyrin repeats located within the extensive amine end (NH_2), and the TRP box located in the proximal carboxyl end (COOH).

Figure 2. Representative schematic of TRPM8.

Figure 3. Representative schematic of TRPV1.

Figure 4. Representative schematic of TRPV4.

TRPA1
universal chemo-irritant receptor



Roles in:

Nociceptive Pain

Inflammatory Pain

Hereditary Episodic Pain Syndrome

Headaches/Migraine

Itch

Agonist:

Mustard Oil

Cinnamaldehyde

Allicin

4-Hydroxynonenal

Positive Regulation:

Bradykinin

Chloroquine

Endothelin

Bile acid

Serotonin

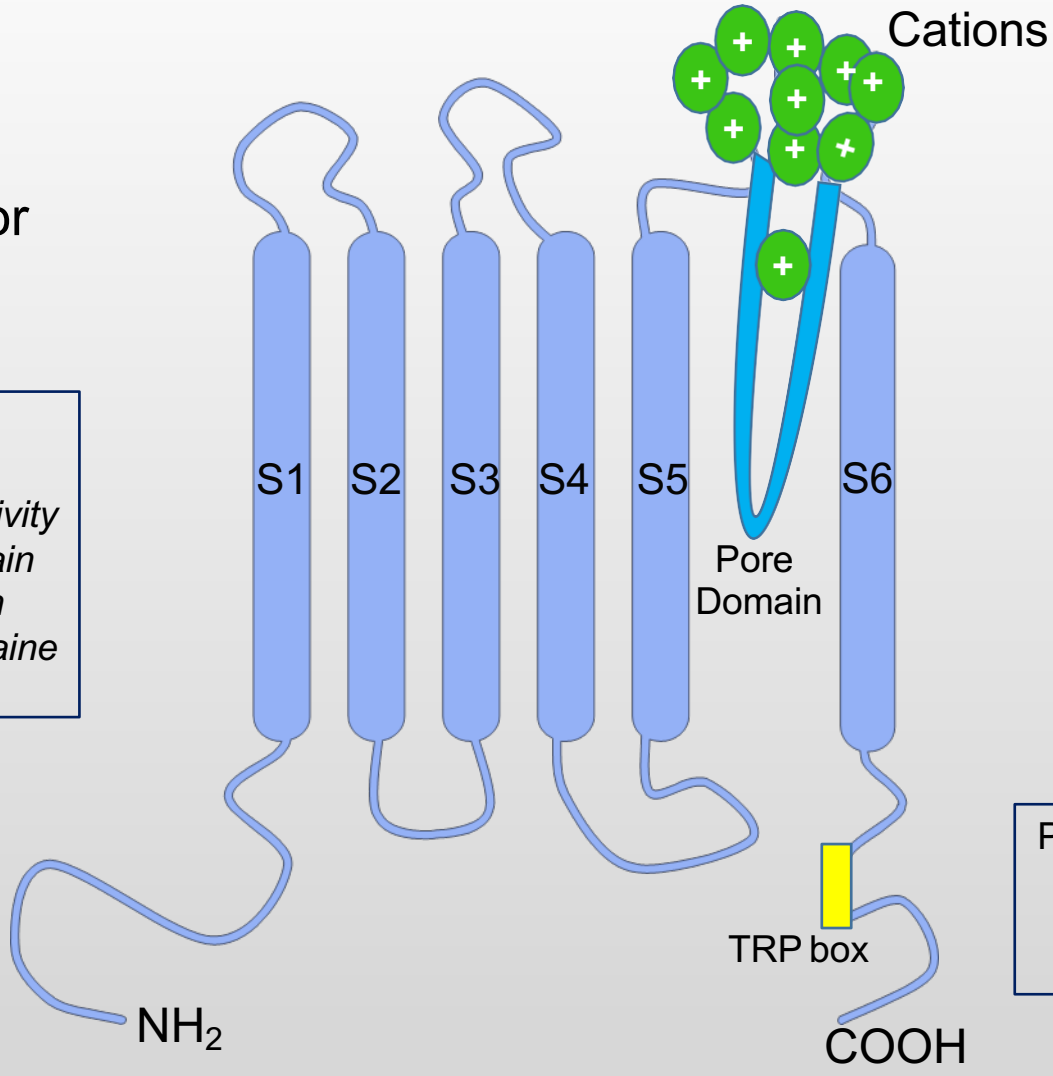
TSLP

LPA

Figure 1

TRPM8

Cold Sensor – menthol receptor



Roles in:

Cold hypersensitivity
Neuropathic Pain
Orofacial Pain
Headaches/Migraine
Itch

Agonists:

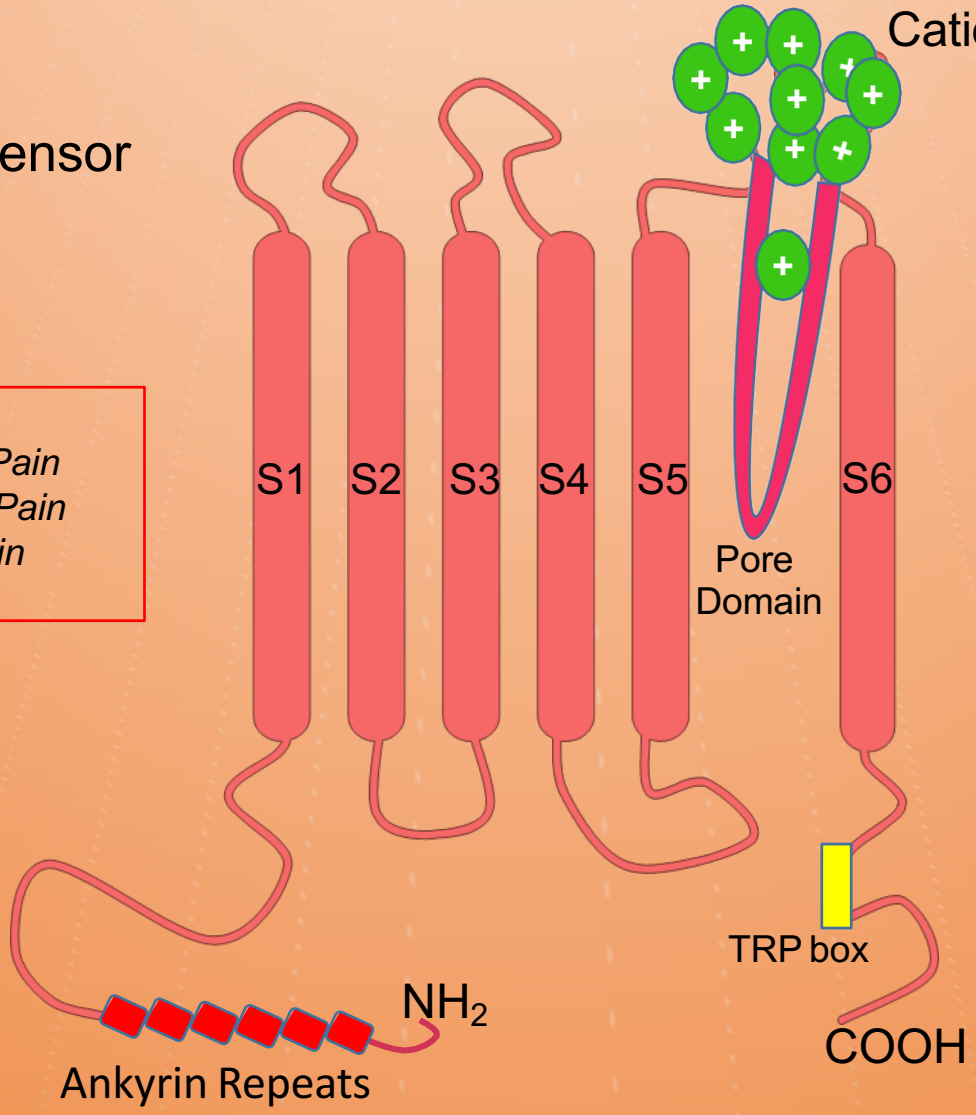
Menthol
Icilin
PUFAs
LPC

Positive Regulation:

Artemin
NGF

Figure 2

TRPV1
Noxious Heat Sensor



Roles in:
Neuropathic Pain
Inflammatory Pain
Visceral Pain
Itch

Agonist:

Pain

Capsaicin
Allicin
LPA
Anandamide

Itch

Imiquimod
Histamine
IL-31

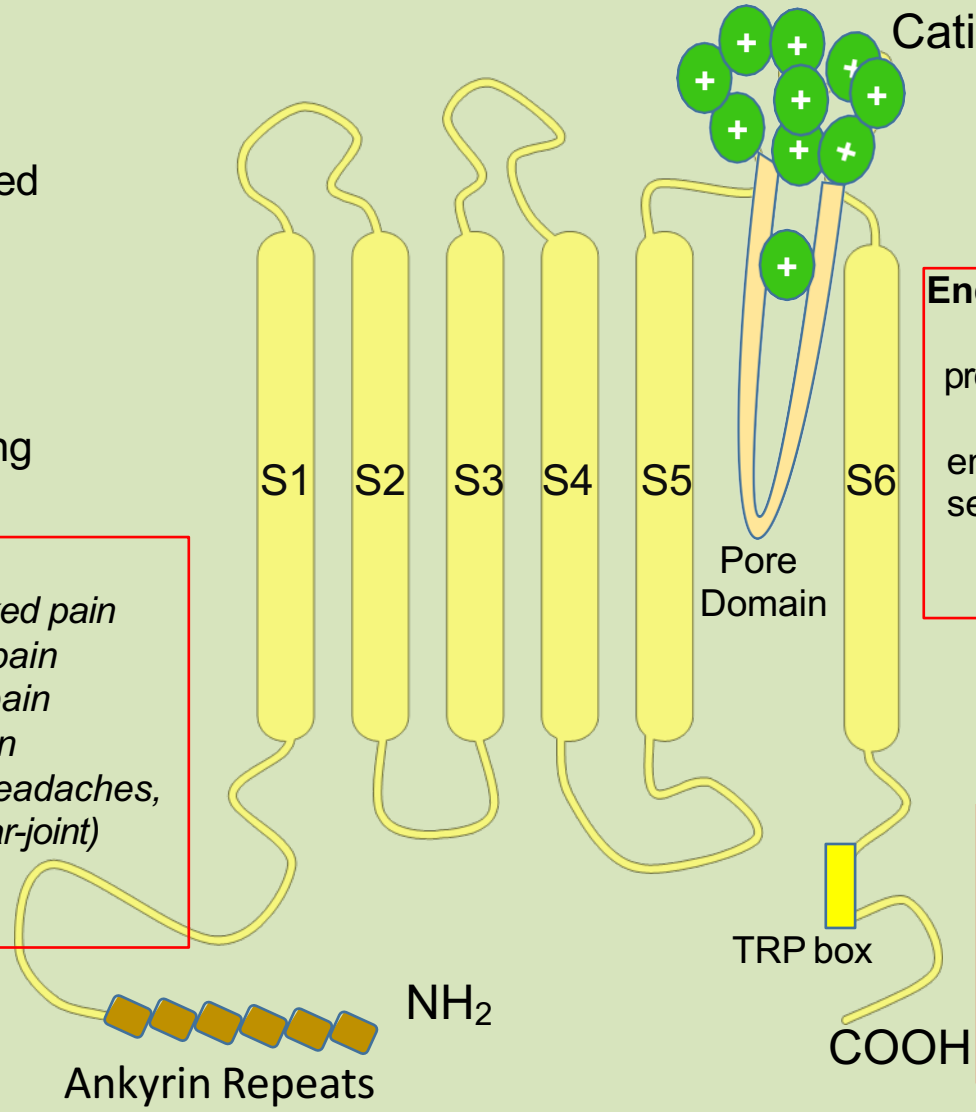
Positive Regulation:
PKA
PKC
NGF
PLA_2

Figure 3

TRPV4

Tonicity-activated
Mechanically-activated
UVB-activated
Warmth-modulated

PAR-2 signaling
IL17-R signaling
Endothelin-R signaling



Endogenous agonist/modulator
pro-pain prostanoid
e.g. 5,6-EET, PGE2
endothelin
serotonin

Roles in:
Mechanically-evoked pain
Inflammatory pain
Neuropathic pain
Visceral pain
Trigeminal pain (incl headaches, temporo-mandibular-joint)
Itch

2nd messenger systems that can activate TRPV4:
PKC
PLC
PI3K

Figure 4

Osmomechanical-sensitive TRPV channels in mammals

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Running title: TRPV channels in osmo and mechanosensation

Key words: TRP, TRPV, osmotic stimuli, mechanical stimuli, osmotransduction, mechanotransduction

Contents

1. Abstract

Osmo-mechano-sensitive channels function to enable cells to sense and transduce stimuli on the membrane. Transient receptor potential (TRP) ion channels have been identified to respond to diverse external and internal stimuli, amongst them osmotic and mechanical stimuli. The “osmo- and mechano-TRPs” are members of the TRPV, TRPC, TRPA, TRPP, and TRPM subfamilies. This chapter will summarize findings on the TRPV subfamily in vertebrates. Of the six mammalian TRPV channels, TRPV1, 2 and 4 were demonstrated to function in transduction of osmotic and/ or mechanical stimuli at the cellular as well as the organismal levels.

2. Introduction: The TRPV subfamily

Within the TRP superfamily of ion channels (Cosens and Manning 1969) (Montell and Rubin 1989) (Wong et al. 1989) (Hardie and Minke 1992) (Zhu et al. 1995), the TRPV subfamily stepped into the lime-light in 1997 (Colbert, Smith, and Bargmann 1997, Caterina et al. 1997), when its founding members, OSM-9 in *C.elegans* and TRPV1 in mammals, were first reported. OSM-9 was identified through genetic screening for worms’ defects in osmotic avoidance (Colbert, Smith, and Bargmann 1997). TRPV1 was identified by an expression cloning strategy (Caterina et al. 1997) (this is also true for TRPV5 and 6 which will not be discussed in this chapter because, up to now, they have not been implicated in osmotic and mechanical signaling). TRPV2, -V3, -V4 were identified by a candidate gene approach, respectively (Caterina and Julius 1999, Peier et al. 2002, Gunthorpe et al. 2002, Xu et al. 2002, Kanzaki et al. 1999, Liedtke et al. 2000, Strotmann et al. 2000, Wissenbach et al. 2000). The latter strategy also led to

the identification of four additional *C.elegans ocr* genes (Tobin et al. 2002) and two *Drosophila trpv* genes, Nanchung (NAN) and Inactive (IAV) (Kim et al. 2003, Gong et al. 2004). The TRPV channels can be sub-grouped into four branches by sequence comparison. One branch includes four members of mammalian TRPVs, TRPV1, -V2, -V3, and -V4; in vitro whole cell recording showed that they respond to temperatures higher than 42, 52, 31, and 27°C, respectively, suggesting that they are involved in thermosensation, hence the term “thermo-TRPs”. Illuminating review articles on “thermo-TRPs” are available for in-depth reading (Clapham 2003, Patapoutian 2005, Tominaga and Caterina 2004, Caterina and Julius 1999, Caterina and Montell 2005). The second mammalian branch includes the Ca^{2+}-selective channels, TRPV5 and TRPV6, possibly subserving Ca^{2+} uptake in the kidney and intestine (Hoenderop et al. 1999, den Dekker et al. 2003, Peng et al. 1999, Peng, Brown, and Hediger 2003). One invertebrate branch includes *C.elegans* OSM-9 and *Drosophila* IAV; the other branch comprises OCR-1 to -4 in *C.elegans* and *Drosophila* NAN.

This chapter will elucidate the role of mammalian TRPV channels in signal transduction in response to osmotic and mechanical stimuli, as well as provide comments on selected recent insights regarding other TRP ion channels that respond to osmotic and mechanical cues. These “osmo- and mechano-TRPs” (Liedtke and Kim 2005) are TRPV1 (Liedtke et al. 2000), -2 (Muraki et al. 2003), -4 (Strotmann et al. 2000), (Liedtke et al. 2000), TRPC1, (Chen and Barritt 2003), TRPC3 (Quick et al. 2012), TRPC6 (Spassova et al. 2006), TRPA1 (Corey et al. 2004, Nagata et al. 2005) TRPP2 (Nauli et al. 2003), -3 (Murakami et al. 2005), and TRPM3 (Grimm et al. 2003), -4 (Earley, Waldron, and Brayden 2004), -7 (Numata, Shimizu, and Okada 2007) and TPML3 (Di

Palma et al. 2002). A full listing of mammalian TRPs involved in osmomechanosensation can be found in table 1.

3. TRPV1

Sharif Naeini *et al*., reported that *Trpv*1$^{-/-}$ mice failed to express an N-terminal variant of the *Trpv1* gene in magnocellular neurons, known to be osmotically sensitive, of the supraoptic and paraventricular nucleus of the hypothalamus (Naeini et al. 2005). As these osmo-sensory neurons are known to secrete vasopressin, the *Trpv*1$^{-/-}$ mice were found to have an impaired ADH secretion in response to systemic hypertonic stimuli, and their magnocellular neurons did not show an appropriate electrical response to hypertonicity (Naeini et al. 2005). These findings made Bourque and colleagues reason that this *Trpv1* N-terminal variant was very likely involved as (part of) a tonicity sensor of intrinsically osmo-sensitive magnocellular neurons. The respective cDNA was indeed cloned from mouse in the Liedtke-Lab, then from rat in Bourque's lab, and further characterized by Bourque's group as a TRPV1 ion channel, non-capsaicin receptor, and responsive to hypertonicity and also thermal cues in the vicinity of 37˚C, published recently (Zaelzer et al. 2015). In keeping with the latter identification of osmotically-thermally sensitive non-vanilloid receptor TRPV1 channels, hyperosmolality activated TRPV1 in vasopressin neurons of rat supraoptic nucleus (SON), resulted in cell depolarization and Ca^{2+} entry through TRPV1 channels themselves and voltage-dependent Ca^{2+} channels suggesting an osmo-sensing role of TRPV1 in these neurons (Moriya et al. 2015) .

In terms of regulation of renal function, TRPV1-mediated mechanosensation in the rat kidney has been attributed to H_2O_2 generated by NADPH oxidase 4 (Nox4). H_2O_2 augmented the release of substance P (SP) from kidney-innervating sensory neurons by enhancing the activity of Ca^{2+}-permeable TRPV1 channels (capsaicin receptors). Both TRPV1 and neurokinin-1 receptor activation contributed to increases in afferent renal nerve activity (ARNA) after mechanostimulation (Lin et al. 2015). The function of Nox4 in renal mechanosensitive innervating nerve fibers has a profound effect on the reflex control of urinary excretion, because Nox inhibition attenuates H_2O_2 production and diuretic/natriuretic responses in the renorenal reflex and in response to saline loading (Lin et al. 2015)

Hypertonic sensing TRPV1 channels found in human conjunctival epithelial cells, where TRPV1 was involved in Ca^{2+} regulation of volume (Mergler et al. 2012), possibly based on the hypersensitivity-gated TRPV1 variant identified by Liedtke-Bourque (Zaelzer et al. 2015).

4. TRPV2

In heterologous cellular systems, TRPV2 was initially described as a temperature-gated ionotropic receptor for stimuli > 52^{o}C (Caterina and Julius 1999). TRPV2 was also demonstrated to respond to hypotonicity and mechanical stimulation (Muraki et al. 2003). Heterologously expressed TRPV2 in CHO cells displayed a similar response to hypotonicity. These cells were also subjected to stretch by applying negative pressure to the patch-pipette and by stretching the cell membrane on a mechanical stimulator.

Both maneuvers led to Ca^{2+} influx that was dependent on heterologous TRPV2 expression. Arterial smooth muscle cells from various arteries expressed TRPV2. These myocytes responded to hypotonic stimulation with Ca^{2+} influx. This activation could be reduced by specific down-regulation of TRPV2 protein by an anti-sense strategy (Muraki et al. 2003).

Recently, TRPV2 was implicated in osmosensation in skeletal muscle fibers (Zanou et al. 2015). The response to hyperosmotic shock in normal muscle fibers and in muscle fibers expressing a dominant negative mutant of the TRPV2 channel (TRPV2-DN) was investigated. Hyperosmotic shock induced TRPV2 activation, which accelerated muscle cell depolarization and allowed the subsequent Ca^{2+}release from the sarcoplasmic reticulum, activation of the Na(+) -K(+) -Cl(-) cotransporter by SPAK (Step2-related Proline Alanine-rich kinase), and the regulatory volume increase (RVI) response. In TRPV2-DN cells, slower membrane depolarization, loss of the Ca^{2+} transients and RVI was reported in response to hyperosmotic shock. (Zanou et al. 2015).

TRPV2 has been implicated as a candidate stretch-activated channel in myocyte intercalated discs, and for the mechanical stimulation-dependent Ca^{2+} signaling of cardiomyocytes, required in the maintenance of cardiac structure and function (Katanosaka et al. 2014). Using cardiac-specific TRPV2-knockout mice, cardiac-specific TRPV2 elimination led to a severe decline in the heart's pump function with the disorganization of the intercalated disc structure, conduction defects and increased mortality. Loss of TRPV2 resulted in neonatal cardiomyocytes with no intercalated discs, showed no intracellular Ca^{2+} increase upon stretch stimulation. TRPV2-deficient hearts showed downregulation of insulin-like growth factor-1 (IGF-1)

receptor/PI3K/Akt signalling. InTRPV2-deficient hearts, IGF-1 administration partially prevented chamber dilation and improved cardiac pump function.

In odontoblasts, TRPV2 was activated by extracellular hypotonicity, resulting in Ca^{2+} influx and inwards currents, which were inhibited using TPRV2 antagonist tranilast and tetraethylammonium-chloride (TEA) (Sato et al. 2013).

TRPV2 has been implicated by Shibasaki et al, to be involved in the regulation of axonal outgrowth. TRPV2 expression was found in spinal motor neurons, dorsal root ganglia (DRG), axonal shafts and growth cones (Shibasaki et al. 2010). When TRPV2 was activated in a membrane stretch-dependent manner, it promoted axon outgrowth. Knockdown of TRPV2 using a dominant-negative TRPV2 and TRPV2-specific shRNA inhibited axonal outgrowth (Shibasaki et al. 2010).

Mahari et al studied the contribution of TRPV2 in the stomach as it responds to mechanical stimuli associated with food intake by looking at the contribution of TRPV2 to gastric adaptive relaxation (GAR) and gastric emptying (GE) (Mihara et al. 2013). *Trpv2* mRNA was detected throughout the mouse gastrointestinal tract and myenteric neurons in the stomach. GAR, which was expressed as the rate of decline of intragastric pressure in response to volume stimuli, was significantly enhanced by the TRPV2 activating probenecid, and the effect was inhibited by the TRPV2 inhibiting tranilast. Gastric emptying (GE) was significantly accelerated by TRPV2 agonist applications, and the enhancement was significantly inhibited by inhibitor co-application (Mihara et al. 2013). However, these compounds lack specificity for TRPV2 channels and therefore final doubts about these data remain.

TRPV3 has not (yet) been characterized as osmo-mechano-TRP, neither in heterologous systems nor in live animals or human studies. The same is true for TRPV5 and TRPV6.

5. TRPV4

CHO cells responded to hypotonic solution when they were (stably) transfected with TRPV4 (Liedtke et al. 2000). HEK-293T cells, when maintained by the same authors, were found to harbor *Trpv4* cDNA, which was cloned from these cells. However, *trpv4* cDNA was not found in other batches of HEK 293T cells, so that this cell line was used as heterologous expression vehicle by other groups (Strotmann et al. 2000, Wissenbach et al. 2000). Notably, when comparing the two settings it was obvious that the single-channel conductance was different (Liedtke et al. 2000, Strotmann et al. 2000). This underscores the relevance of gene expression in heterologous cellular systems for the functioning of TRPV4 in response to a basic biophysical stimulus. Also, it was found that the sensitivity of TRPV4 could be tuned by warming of the media. Peak sensitivity of gating in response to hypotonicity was recorded at core body temperature of the respective organism, and TRPV4 channels from both birds (chick, core body temp. 40^{o}C) and mammals (rat, 37 ^{o}C) were compared, again in CHO cells (Liedtke et al. 2000). Similar results were found in another investigation with mammalian TRPV4 in HEK-293T cells (Gao, Wu, and O'Neil 2003). Later work by Ching Kung's group unambiguously suggests that TRPV4 is critically and closely involved in mechanotransduction (Loukin, Su, and Kung 2009). Other work by Thodeti and Ingber strongly argues along the same lines (Matthews et al. 2010).

In addition, in the earlier study by Gao and O'Neil, the cells were mechanically stretched, without a change of tonicity. At room temperature, there was no response upon mechanical stretch, however, at 37^{o}C the isotonic response to stretch resulted in the maximum Ca^{2+} influx of all conditions tested. In two other investigations, TRPV4 was found to be responsive to changes in temperature (Guler et al. 2002, Watanabe et al. 2002). Temperature change was accomplished by heating the streaming bath solution. However, flow is a mechanical stimulus, presenting as mechanical shear stress to the cell. Gating of TRPV4 was amplified when hypotonic solution was used as streaming bath. In one investigation, temperature stimulation could not activate the TRPV4 channel in cell-detached inside-out patches (Watanabe et al. 2002). With respect to the gating mechanism of TRPV4 in response to hypotonicity, two other investigations report conflicting results on phosphorylation sites of TRPV4 that are necessary for the response to hypotonicity. One study reported that TRPV4 was tyrosine-phosphorylated in HEK-293T cells and in distal convoluted tubule cells from mouse kidney (Xu et al. 2003, Tian et al. 2004). Tyrosine phosphorylation was sensitive to specific inhibition of the Src family tyrosine kinases. The Lyn tyrosine kinase was found to coimmunoprecipitate TRPV4 protein and to bear a prominent role in phosphorylation of TRPV4 (Y253). A point mutation of Y253 greatly reduced hypotonicity-induced gating. On the other hand, in another investigation, in HEK-293T cells, hypotonicity activated TRPV4 by phospholipase-A2 mediated formation of arachidonic acid via a cytochrome P450 epoxygenase pathway (Vriens et al. 2003). In HEK cells, this signaling mechanism did not apply for gating of TRPV4 by increased temperature or by the non-phosphorylating phorbol-ester 4-alpha PDD. This latter

activation mechanism was reported to be dependent on phosphorylation of Y555. However, the authors of this study could not replicate the aforementioned finding, namely that tyrosine kinase phosphorylation of Y253 of TRPV4 was critical for hypotonicity-induced gating. - Why this divergence? The discrepancy re-iterates the pivotal role of the host cell in heterologous expression experiments.

In the mammalian oviduct, ciliary beat frequency of ciliated cells was found to be influenced by gating of TRPV4 (Andrade et al. 2005). In explanted ciliated cells, and also in heterologously transfected HeLa cells, TRPV4 could be activated (mechanically) by exposing the cells to hyperviscous, isotonic media. In interesting follow-up studies, TRPV4 was found to function as an ionotropic receptor for particulate matter air pollution from combustion engines in human bronchial epithelia, where it regulated a pro-inflammatory and proteolytic phenotype, capable of enhancing chronic obstructive pulmonary disease (COPD) (Li et al. 2011).

TRPV4 also has been found to play a role in maintenance of cellular osmotic homeostasis. One particular cellular defense mechanism of cellular osmotic homeostasis is regulatory volume change, namely regulatory volume decrease (RVD) in response to hypotonicity and regulatory volume increase (RVI) in response to hypertonicity. Valverde's group published that TRPV4 mediates the cell-swelling induced Ca^{2+} influx into bronchial epithelial cells that triggers RVD via Ca^{2+}-dependent potassium channels (Arniges et al. 2004). This cell swelling response did not work in cystic fibrosis (CFTR) bronchial epithelia, where, on the other hand, TRPV4 could be activated by 4-alpha-PDD, leading to Ca^{2+} influx. Thus, in CFTR bronchial epithelia, RVD could not be elicited by hypotonicity, but by 4-alpha-PDD. In yet another

investigation, Ambudkar and colleagues found the concerted interaction of aquaporin 5 (AQP-5) with TRPV4 in hypotonic swelling-induced RVD of salivary gland epithelia (Ciura, Liedtke, and Bourque 2011). These findings shed light on mechanisms operative in secretory epithelia (such as salivary, tear, sweat, pancreatic and intestinal glands and airway) that underlie watery secretion based on a concerted interaction of TRPV4 and AQP-5.

TRPV4 was found to be a key osmosensing component in airway sensory nerve reflexes, as it induced activation of guinea pig airway–specific primary nodose ganglion cells. TRPV4 activators and hypo-osmotic solutions caused depolarization of murine, guinea pig, and human vagus and firing of Aδ-fibers (not C-fibers), which was inhibited by TRPV4 and P2X3 receptor antagonists. Both antagonists blocked TRPV4-induced cough (Bonvini et al. 2016). TRPV4 was implicated as a novel therapeutic target for neuronal hyper-responsiveness in the airways and symptoms, such as chronic cough. Chronic cough can be viewed as an airway manifestation of a neural hypersensitivity.

In regards to pain, in *Trpv*4$^{-/-}$ mice, the response to noxious mechanical, not noxious thermal stimulation is diminished (Liedtke and Friedman 2003). In the absence of TRPV4, which in wild-type mice could be shown to be expressed in sensory ganglia (Liedtke and Friedman 2003, Liedtke et al. 2000, Chen et al. 2014, Chen et al. 2013) and, in skin, in subcutaneous nerve fibers and keratinocytes (Delany et al. 2001, Guler et al. 2002), the threshold to noxious mechanical stimulation was significantly elevated. Earlier follow-up studies, particularly those by Jon Levine's and Nigel Bunnett's groups were characterized in an earlier TRP book edited by Liedtke and Heller, and will not be rehearsed here (Liedtke Editor, 2007).

As for thirst and central osmoregulation, earlier work on the role of TRPV4 in thirst and central osmoregulation was reflected in the earlier TRP book, and will also not be rehearsed here. More recent work appears to suggest a role for TRPV4 in thirst and central osmoregulation, which will need to be resolved more clearly by tissue- or cell-lineage specific and inducible knockouts of the channel (Ciura, Liedtke, and Bourque 2011, Janas et al. 2016, Sakuta et al. 2016).

In regards to TRPV4's function in the skeleton, TRPV4 is highly expressed in articular chondrocytes where the channel responds to osmotic cues (Phan et al. 2009). In humans, TRPV4 mutations that alter the function of the channel disrupt normal skeletal development and joint health (Leddy et al. 2014) (Nilius and Voets 2013). In mice, *Trpv4* pan-null deletions resulted in a lack of osmotically induced Ca^{2+} signaling in articular chondrocytes, plus a progressive, sex-dependent increase in bone density and osteoarthritic joint degeneration (Clark et al. 2010). In another recent study by the Liedtke-Guilak group, using a tissue-specific, inducible*Trpv4* gene-targeted mice, it was demonstrated that loss of chondrocyte TRPV4 resulted in a loss of TRPV4-mediated cartilage mechanotransduction in adulthood, and a reduction of the severity of aging-associated osteoarthritis (OA), not post-traumatic OA. They also noted cartilage-specific deletion of *Trpv4* showed a decrease in total joint bone volume and a decreased osteophytes in the joint (O'Conor et al. 2016).

In another recent study, O'Connor et al., revealed that upon mechanical stimulation, chondrocytes responded in a TRPV4-dependent manner, which involved the transcriptional enhancement of anabolic growth factor gene expression and inhibition of proinflammatory mediators. Inhibition of TRPV4 with GSK205 blocked both the

compositional and functional augmentation of mechanically loaded agarose-embedded chondrocytes constructs, further supporting the role of TRPV4-mediated mechanotransduction in response to mild mechanical stress in regulating chondrocyte matrix metabolism toward an anabolic phenotype (O'Conor et al. 2014). Activation of TRPV4 with GSK101 in the absence of mechanical loading, similarly enhanced anabolic and suppressed catabolic gene expression and potently enhanced matrix accumulation and functional properties of the chondrocyte-agarose constructs, and potently increased cartilage matrix biosynthesis. This opens the door for tissue engineering, following a molecular logic of TRPV4 signaling in cartilage.

In an attempt to decipher the mechanism, Kobayakawa et al., showed that in human chondrocytic cells, excessive mechanical stress loading induced the metalloproteinase ADAM10 expression and enhanced CD44 (a cell-surface glycoprotein) cleavage which can lead to the loss of extracellular matrices in chondrocytes. Chemical inhibition of TRPV4 significantly suppressed mechanical stress induced ADAM10 expression and CD44 cleavage. Conversely, chemical activation of TRPV4 increased ADAM10 expression and enhanced CD44 cleavage. Such finding suggests a mechanism whereby mechanical loading significantly increases the expression of ADAM10, which in turn enhances CD44 cleavage in HCS-2/8 cells, with TRPV4 mechanoreceptor mediating this process (Kobayakawa et al. 2016). These findings corroborate the Liedtke-Guilak concept (O'Conor et al. 2016) that TRPV4-mediated Ca^{2+} signaling in chondrocytes plays a central role in the transduction of mechanical signals to support cartilage extracellular matrix maintenance and joint health.

In the vertebrate eye, TRPV4 is involved in mechanical intra-ocular pressure sensing and its regulation. TRPV4 was found in human conjunctival epithelial cells, where it was involved in Ca^{2+} regulation of volume (Mergler et al. 2012). TRPV4 was observed to regulate Ca^{2+} homeostasis, cytoskeletal remodeling, conventional outflow and intraocular pressure (IOP) in the mammalian eye (Ryskamp et al. 2016). Mechanical force was applied to trabecular meshwork (TM) cells, which resulted in sustained, stretch-dependent Ca^{2+} elevations that could be mimicked by GSK101 and suppressed by TRPV4 blocker. Systemic delivery, intraocular injection or topical application of putative TRPV4 antagonist prodrug analogs lowered IOP in glaucomatous mouse eyes and protected retinal neurons from IOP-induced death. It was observed that phospholipase A2 (PLA2) antagonists inhibit stretch-induced Ca^{2+} signals and that TRPV4 blockers suppress AA-induced Ca^{2+} increases suggesting that the channel is activated through the canonical pathway. A mechanism proposed was that mechanical stress (e.g., pressure, swelling, and tissue distension) stretches the plasma membrane and activates TRPV4 and a Ca^{2+}- and stretch-sensitive PLA2. The product, arachidonic acid (AA), leads to the synthesis of eicosanoid metabolites (EETs), which activates TRPV4. Stretch might activate PLA2 simultaneously with TRPV4 or alternatively, stretch-induced TRPV4 activation could stimulate Ca^{2+}-dependent PLA2s which amplify the initial TRPV4 signal. This study implicates TRPV4 as a potential IOP sensor within the conventional outflow pathway and as a novel target for treating ocular hypertension. Future studies will have to deconstruct ocular TRPV4 function using cell- and lineage specific and inducible knockout models.

6. Outlook for future applications involving TRP ion channels

Targeting osmo-mechano TRPs for treatment of human disease has become a more compelling rationale since the field has been founded (Kanju et al. 2016, Brierley et al. 2009). Basic science progress as well as translational advances, such as those communicated in the latter study, have nourished this development. In particular the arenas of pain and inflammation (Wilson et al. 2013, D'Aldebert et al. 2011, Engel et al. 2011, Bonvini et al. 2015), skeletal patho-physiology and disease (McNulty et al. 2015), and cardio-vascular patho-physiology and disease (White et al. 2016), appear to be areas-of-interest where non-incremental progress toward rationally-guided medical diagnoses, prevention and treatments could be imminent.

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Table 1. Mammalian TRP Channels involved in osmo and mechanosensation

TRPs	Mechano-activation	Osmo-activation	Critical expression	References
TRPA1	-	hypertonicity	sensory neurons	(Corey et al. 2004, Nagata et al. 2005)
TRPC1	stretch	hypotonicity	CHO cells, blood vessels, DRG, liver	(Chen and Barritt 2003)
TRPC3	stretch	-	sensory neurons	(Quick et al. 2012)
TRPC4	-	-	-	-
TRPC5	stretch	hypotonicity	HEK293	(Gomis et al. 2008)
TRPC6	stretch	-	CHO cells, blood vessels, DRG, kidney	(Spassova et al. 2006), (Quick et al. 2012)
TRPC7	-	-	-	-
TRPM1	-	-	-	-
TRPM2	-	-	-	-
TRPM3	-	hypotonicity	kidney	(Grimm et al. 2003)
TRPM4	stretch	-	vascular smooth muscle	(Earley, Waldron, and Brayden 2004)
TRPM5	-	-	-	-
TRPM6	-	-	-	-
TRPM7	stretch, shear stress	hypotonicity	-	{Numata, 2007 #127
TRPML1	-	-	-	-
TRPML2	-	-	-	-
TRPML3	shear stress, stretch	-	hair cells of the ear	(Di Palma et al. 2002).
TRPP2	shear stress	-	endothelial cells, kidney	(Nauli et al. 2003)
TRPP3	-	hypotonicity	Endoplasmic reticulum, HEK293	(Murakami et al. 2005)
TRPP5	-	-	-	-
TRPV1	stretch	hypertonicity	eye epithelial, kidney , neurons	(Liedtke et al. 2000), (Zaelzer et al. 2015)
TRPV2	stretch, shear stress	hypotonicity	CHO, HEK293, skeletal and cardiac muscle, stomach, neurons	(Muraki et al. 2003), (Shibasaki et al. 2010) (Mihara et al. 2013)
TRPV3	-	-	-	-
TRPV4	stretch, shear stress	hypotonicity	CHO, HEK293, skeletal and cardiac muscle, sensory neurons, airway, bone, chondrocytes, eye	(Strotmann et al. 2000), (Liedtke et al. 2000) (O'Conor et al. 2016) (Clark et al. 2010)
TRPV5	-	-	-	-
TRPV6	-	-	-	-

8. References

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